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Transduction in Mycobacterium Phlei

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TRANSDUCTION IN *MYCOBACTERIUM PHLEI*

by

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LIST OF ABBREVIATIONS AND SYMBOLS

Bo1	=	*Mycobacterium smegmatis* phage Bo1
Bo2	=	*Mycobacterium phlei* phage Bo2
Bo2·F89	=	phage Bo2 last propagated on *M. phlei* F89
Bo2·SN109	=	phage Bo2 last propagated on *M. phlei* SN109
CFU	=	colony forming units
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
HFT	=	high frequency transduction
HIB	=	heart infusion broth
INH	=	isonicotinic acid hydrazide, an anti-tuberculosis drug
LFT	=	low frequency transduction
MM	=	minimal medium suggested by R. Bönicke (see Materials and Methods)
MM'	=	MM modified by addition of ferric chloride and streptomycin and omission of bromcresol purple (see Materials and Methods)
RNA	=	ribonucleic acid
RNase	=	ribonuclease
SSC	=	0.15 M sodium chloride and 0.015 M sodium citrate buffer
str-r	=	resistant to over 50 µg/ml streptomycin
str-s	=	sensitive to 10 µg/ml streptomycin
Tween-80	=	registered trademark for polyoxyethylene sorbitan monooleate (Eastman, Rochester, N.Y.)
xyl^+	=	able to utilize D-xylose as a sole carbon source
xyl^-	=	unable to utilize D-xylose as a sole carbon source
λ	=	*Escherichia coli* phage λ, from *E. coli* K12(λ)

INTRODUCTION

Transfer of genetic information in bacteria, called transformation was first described by Griffith, 1928. He speculated that his transforming principle might be a polysaccharide which is needed as a primer in autocatalytic polysaccharide synthesis, or, possibly a protein which activates the hereditary reaction. The fundamental concept that the "transforming principle" controlled heredity directly, eluded him (Hotchkiss, 1966). His finding paved the way for Avery et al. (1944) to discover that deoxyribonucleic acid (DNA) is the genetic material in pneumococci.

Transformation has been found to occur in a wide variety of bacterial genera: in *Pneumococcus*, (Griffith, 1928); *Hemophilus*, and *Neisseria* (Alexander and Leidy, 1950; Alexander and Redman, 1953); *Bacillus* (Anagnostopoulos and Spizizen, 1961); *Streptomyces* (Jarai, 1961); *Streptococcus* (Perry and Slade, 1962); *Rhizobium* (Balassa, 1955); and *Mycobacterium* (Juhasz et al., 1971). Although transformation in *Mycobacterium* was first reported by Katunuma and Nakasato (1954) and more recently by Tsukamura, Hashimoto and Noda (1960), their work could not be confirmed in repeated attempts by many prominent investigators (Tarnok and Bönicke, 1969).

Transformation by bacteriophage DNA called transfection was first reported by Kaiser and Hogness (1960) in *Escherichia coli* and since then by Romig (1962) in *Bacillus subtilis*, by Harm and Ruppert (1963) in *Hemophilus influenzae* and by Tokunaga and Sellers (1964) in *Mycobacterium smegmatis*. DeSalle (1971) working in our laboratory has shown that DNA from mycobacteriophage phlei Bo2 can transfect *M. phlei*.

Genetic transfer can occur by methods other than by transfer of free DNA. Transfer of DNA requiring intimate cell to cell contact called conjugation (Curtiss, 1969) was first discovered in *Escherichia coli* K12 by Lederberg and Tatum (1946) and later shown to occur in a wide variety of other gram negative bacteria: in *Pseudomonas* by Holloway (1955, 1956); in *Serratia* by Belser and Bunting (1956); in *Vibrio* by Bhaskaran and Iyer (1961); in *Salmonella* by Zinder (1960a); in *Shigella* by Akiba et al. (1960); in *Enterobacter* by deGraff et al. (1968); in *Pasteurella* by Lawton, Morris and Burrows (1968); and in *Rhizhobium* by Heumann (1968). Intergeneric conjugation has also been shown to occur between *Escherichia* and *Shigella* by Luria and Burrous (1957), between *Escherichia* and *Salmonella* by Zinder (1960b); between *Escherichia* and *Serratia* by Falkow et al. (1961); between *Escherichia* and *Pasteurella* by Martin and Jacob (1962) and independently by Ginoza and Matney (1963); and between *Escherichia* and *Proteus* by Falkow et al. (1964). Conjugation has also been demonstrated in the order Actinomycetales which includes the genus *Mycobacterium*. Adams and Bradley (1963) demonstrated conjugation between different species of *Nocardia*, and genetic recombination, believed to be conjugation but not conclusively demonstrated as such (Sermonti and Hopwood, 1960), was first demonstrated in *Streptomyces* by Sermonti and Spada-Sermonti (1955). However, in the genus *Mycobacterium*, conjugation has not yet been detected.

Another method of genetic transfer was discovered inadvertently by Zinder and Lederberg (1952) while investigating genetic transfer they believed to be conjugation in strains of *Salmonella*. Their studies revealed that recombinants could be found not only when cultures of the two strains were

mixed, but also when a culture of one strain was mixed with a cell free extract of the other. They referred to the phenomenon as transduction [transduce: to lead across (Hayes, 1968)] since they hypothesized that some "filterable agent" was carrying genetic information from one strain to the other. They analysed their filterable agent and found it to be a bacteriophage (Zinder, 1955). The discovery of transduction occurred in the same year as the demonstration that the essence of phage infectivity is the injection of its DNA into its host (Hershey and Chase, 1952). Based on this discovery it was quickly hypothesized that during the growth cycle of the phage some DNA of the host was in some way incorporated into the phage particles which thus became vectors transporting it to other bacteria (Zinder, 1955). The phenomenon has since been reported in *Escherichia coli* by Morse (1954), Lennox (1955) and Jacob (1955), to name but a few; in *Shigella* by Lennox (1955) and by Adams and Luria (1958); in *Pseudomonas* by Loutit (1958) and by Holloway and Monk (1960); in *Staphylococcus* by Morse (1959) and by Pattee and Baldwin (1961); in *Proteus* by Coetzee and Sacks (1960); in *Bacillus* by Thorne (1961) and by Takahashi (1961, 1963); in *Vibrio* by Bhaskaran (1958); and in *Rhizobium* by Kowalski (1967).

There are two types of transduction systems. Generalized, or unrestricted transduction, where virtually any loci of the bacterial genome can be transferred, is the typical type with almost all transduction systems fitting this category. Specialized, localized (Hayes, 1968) or restricted transduction, where only one small region of the bacterial chromosome can be transferred, is characteristic of phage λ of *E. coli* and its close relatives, the lambda related (lambdoid) phages, e.g., phages ϕ170, ϕ80, ϕ82, and ϕ84

of *E. coli* (Ozeki and Ikeda, 1968).

The mechanism of transduction is fairly well elucidated. Bacterial DNA is incorporated into the phage head. When these altered phages infect a new host, the bacterial DNA is injected. As in all of the known systems of genetic transfer, once this DNA enters its new host it can recombine with its genome. The mechanics of how the bacterial DNA gets into the phage head is not completely understood. There are two theoretical models, both of which seem well substantiated by experimental evidence. The first classical model can explain both types of transduction systems and seems to be the only explanation for specialized transduction. The phage genome integrates into the bacterial chromosome, as in the case of lysogeny (Lwoff, 1953). Before it separates from the bacterial chromosome and enters the lytic cycle (Lwoff, 1953), recombination between the phage and bacterial genome can occur which results in the replacement of a part of the phage genome by a small part of the bacterial genome. These phages lacking a part of the phage genome are said to be defective. They have, on the other hand, a piece of bacterial genome which they are able to transfer to a future host (Campbell, 1964; Ozeki and Ikeda, 1968). Evidence for this first model came primarily from analysis of the λ system. Morse et al (1956a, 1956b) explained both specialized and generalized transduction by assuming that the phage could either integrate into selected regions of the bacterial chromosome in the case of the former, or into any region in the case of the latter. The study of phage architecture, however, led eventually to the realization that a phage cannot carry both its own DNA and a large piece of foreign DNA. The second model of DNA incorporation is based on the concept that bacterial DNA can be randomly incorporated into the phage head by a process analogous to phenotypic

mixing (Clowes, 1958; Ozeki and Ikeda, 1968). According to this model called the wrapping choice model (Ozeki and Ikeda, 1968), at no time is there any need for physical association between phage and bacterial genomes. Substantiating evidence for this theory came from physical analysis of transducing particles of phage P1 by Ikeda and Tomizawa (1965). The chromosomes of thymine-requiring *E. coli* were density labeled by growth on a medium containing bromouracil and then infected with a virulent mutant of transducing phage P1 in the presence of thymine and radioactive phosphate ($^{32}PO_4$). When the progeny particles, of the transducing lysate, were analysed by density gradient centrifugation, it was found that transducing particles separated in the denser bromouracil band and that these transducing particles were devoid of radioactive label. This indicated that the transducing particles incorporated only fragments of bacterial DNA present at time of infection and contained no phage genome since the phage DNA incorporated thymine and $^{32}PO_4$. This proved that these transducing particles could not have arisen by genetic recombination. The second model better explains how phages such as P1 can transduce almost twice its own weight in foreign DNA.

Transduction systems can be further classified according to the number of transducing particles present in the phage population. If the number is lower than one in 10^6, the phage population is called low frequency transduction (LFT) lysate. If the number is greater than 1 in 10^6, the phage population is called high frequency transduction (HFT) lysate (Morse, 1956a). HFT lysates have been reported for both generalized transducing phages, e.g., P1 an *E. coli-Shigella* phage (Luria et al., 1960), SP-10, a *Bacillus* phage

(Okubo et al., 1963), and for all specialized transducing phages, e.g., coliphage λ (Morse et al., 1956a). In most cases they are obtained from prior transductants by either induction of prophage (Morse et al., 1956a) or by superinfection by a mutant phage (Takahashi, 1961; Luria et al., 1960). There appears to be two mechanisms for high frequency transduction also, based on the models for DNA incorporation discussed earlier. In the case of λ and P1 which are probably the most extensively studied HFT systems, it can be clearly shown that "hybrid formation" is involved. In the case of λ the hybrid is λdg where d means defective and unable to multiply without the aid of a helper phage and g means contains the genetic locus for the ability to ferment galactose (Morse et al., 1956b). In the case of P1 the interpretation of mechanism is complicated by the fact that two genera of bacteria are involved and a generalized transducing phage P1 becomes highly specialized. When P1 from *E. coli* is used to infect strains of *Shigella*, it is possible to transduce the entire lactose (lac) region along with many other characters to *Shigella*, which is totally devoid of any lac genes. Upon superinfection of these transductants with the virulent mutant of P1, P1dl (d=defective; l=lac carrying) is obtained which transfers only the lac region in its entirety, to a new *Shigella* host. The frequency of transfer with λdg HFT or P1dl HFT is about 10^{-2} (Morse et al., 1956b; Luria et al., 1958). In the case of Bacillus phage SP-10, a phage is obtained upon lytic propagation which is capable of transferring any character from its previous host to its new host at a frequency of about 10^{-4}. These transducing particles are found to be nearly devoid of phage DNA (Okubu et al., 1963). It is assumed, therefore, that these particles originate by indiscriminate incorporation of DNA

fragments into the phage head rather than by genetic recombination.

In early reports on transduction, it was assumed that only one character could be transferred by any single transducing particle. Stocker working with Zinder and Lederberg showed that this was not so. He transferred two characters simultaneously using the original Zinder and Lederberg system (Stocker et al., 1953). The transfer of two or more characters by a single phage particle is called co-transduction and can only occur if the markers are sufficiently close to both be incorporated into the phage head. There are phages e.g., coliphage P1 and subtilis phages PBS-1 and SP-10, which can take in as much as 2% of the bacterial genome, which means that they can transfer a segment of DNA many times larger than that which can be transferred by transformation. Using phages of this type it was possible to utilize transduction as a tool for genetic mapping, since the greater the frequency of co-transduction the closer on the genome were the two markers (Hayes, 1968).

Transduction has been used for genetic mapping by many investigators. The first report of genetic mapping was by Demerec and Hartman (1956). They mapped the try, his, met (mutants requiring tryptophan, histidine, and methionine, respectively) loci in *Salmonella typhimurium*. The most extensive map obtained by using transduction is the work of Dubnau et al., (1967). They could map about 30 characters in *B. subtilis* strain 168 using transduction mediated by phage PBS-1 in addition to transformation. When comparing this work to the great accomplishments of Jacob and Wollman and other workers who used conjugation to map *E. coli* and *Salmonella* (Jacob and Wollman, 1961; Taylor, 1970; Sanderson, 1970), one can see that transduction is not very efficient as a tool for genetic mapping. This is due solely to

the size of the chromosome which can be transferred by a phage, at most 5% of the bacterial chromosome, as compared to virtually the entire chromosome which can be transferred by conjugation. However, it must be emphasized that one must use whatever tools one has, and conjugation cannot be demonstrated in all genera of bacteria.

In contrast to the relatively poor value of transducing phages as tools of macro-mapping, i.e., the arrangement of genetic markers on the genome, transducing phages serve as the best tools for micro-mapping, i.e., the arrangement of functional genes within a given genetic locus. This was first demonstrated by Hartman, Loper and Serman (1960) as a follow up of Hartman's initial work, (Demerec and Hartman, 1959). Utilizing over 200 mutants requiring histidine for growth, they could demonstrate by transduction that the seven loci which each determined a specific step in histidine synthesis were for the most part themselves composed of complex loci, i.e., two or more functional genes.

In the genus *Mycobacterium* no well elaborated genetic transfer system had been developed at the time this project was undertaken. Juhasz (1960) reported transfer of streptomycin resistance between *M. phlei* and *M. bovis* strain BCG. He found that culture filtrates of the BCG strain conferred streptomycin resistance on *M. phlei* and that deoxyribonuclease (DNase) did not significantly affect the filtrates' transfer activity. Since it had previously been shown by several investigators that mycobacteria can be naturally lysogenic (Segawa et al., 1960; Hnatko, 1953; Bowman and Redmond, 1959), i.e., they contained a phage genome as part of their host genetic material and had the ability to produce phage without further infection by external phage

(Lwoff, 1953), it seemed logical that the BCG strain employed by Juhasz may have been lysogenic for some transducing phage. Unfortunately no phage could be detected. The problem in this case was that even if a phage were present, the lack of a suitable indicator strain made it impossible to detect its presence (Redmond, 1963; Juhasz et al., 1969). Another report on what was believed to be a transduction system was made by Redmond et al. (1967) but as in the former case, a transducing phage could not be demonstrated. The major breakthrough came when virtually simultaneously Sundar Raj and Ramakrishnan (1970), in India, reported on a transduction system in *Mycobacterium smegmatis* while our group, working independently, described a transduction system in *M. phlei*. The development and elaboration of this system in *M. phlei* comprise the main theme of this dissertation.

Although many mycobacterial phages have been reported in the literature (Redmond, 1963) none have been well characterized. Ever since the initial discovery of phages active on mycobacteria (Gardner and Weiser, 1947), early investigators were primarily interested in the use of phages in phage typing of mycobacterial species (Hauduroy and Rosset, 1948; Hnatko, 1953; Cater and Redmond, 1960 and 1961). For this reason early reports contained very little as far as characteristics of the phages themselves, but consisted mainly of details of the isolation procedure along with the host range of the phages. Therefore, it was necessary to choose our prospective transducing phage randomly from those which were readily available.

The phage ultimately selected was the mycobacteriophage phlei Bo2, initially isolated by Juhasz and Bönicke (1965) and partially characterized by Juhasz and Bönicke (1970). The original designation of the phage was B2 but it

was later redesignated Bo2 to avoid confusion with phage B2 of Japanese investigators (Gelbart et al., 1970). Concurrent with the studies on transduction, characterization of the phage was also undertaken since it was obvious that certain characteristics of both the phage and its host had to be determined before any analysis of the transfer system itself could be made. The only characterization of phage Bo2 that could be found in the literature were electron microscopic morphology studies, plaque morphology studies, host range studies and heat sensitivity studies (Juhasz and Bönicke, 1965, 1966, 1970). The size of the phage as described by Juhasz and Bönicke (1970) was 150 nm average tail length and 49 nm average head diameter. The electron micrographs presented in their paper showed Bo2 as a long, striated, flexible-tailed virus with a spherical-hexagonal head. They also reported that it formed hazy plaques on Froman's agar medium (Juhasz and Bönicke, 1965) but gave no description of the size of plaques. They listed the host range as specific for all strains of *M. phlei* but did obtain a variant which could also lyse *M. smegmatis* (Juhasz and Bönicke, 1970).

The role of phage in bacterial variation is not limited to transduction. Phage infection itself can result in host changes without any actual transfer of genetic information from a previous host. This phenomenon known as phage conversion (Groman, 1960) arises from the fact that the phage genome itself can in some instances code for new host characteristics. While this phenomenon had not been conclusively demonstrated in mycobacteria, it has been shown that quantitative changes in existing characters can be produced by phage infection in this genus (Bönicke, 1967; Juhasz et al., 1969; Mankiewicz et al., 1969; Gelbart et al., 1970).

The purpose of this dissertation project was to develop a transduction system in *M. phlei* and to elaborate some facets of the system.

MATERIALS AND METHODS

A. *Bacterial strains.* *Mycobacterium phlei* was selected as the species for transduction experiments. Eighteen strains were examined for suitable markers which could be used in future experiments. The following strains were tested: SN101-SN115, SN118, and SN119 of the Borstel Culture Collection, Borstel Research Institute for Experimental Biology and Medicine, Borstel, West Germany, and F89 originally obtained from S. Froman, Olive View Hospital, Olive View, California. *M. smegmatis* strains SN2 and SN46 also of the Borstel Collection, and a strain of *M. smegmatis* and its auxotrophs, obtained from K.T. Holland, University of Leeds, Leeds, England, were also employed in these studies.

B. *Bacteriophage.* *Mycobacteriophage phlei* Bo2 (hereafter referred to as phage Bo2), originally isolated by Juhasz and Bönicke (1965) from dung, was obtained from the Borstel Culture Collection. *Mycobacterium phlei* strains SN109 and F89 (hereafter referred to as SN109 and F89) were used as the propagating hosts. Mycobacteriophage smegmatis Bo1, originally isolated from soil by Juhasz and Bönicke (1965), was also obtained from the Borstel Culture Collection. *Mycobacterium smegmatis* strain SN2 was used as the propagating host for this phage.

C. *Media.* Bacterial strains were propagated on Löwenstein-Jensen plates (Colab, Glenwood, Illinois) and maintained on Löwenstein-Jensen slants (Colab) Bacteria were assayed by viable counts on 1.5% nutrient agar (Difco, Detroit, Michigan) plates; the agar overlay was prepared from nutrient broth (Difco) by the addition of 0.7% Bacto-agar (Difco). Suspensions of bacteria

were made in heart infusion broth (HIB, Difco). Nutrient agar (Difco, 1.5%) was used for purification of bacterial strains. Phage Bo2 was propagated on its *M. phlei* host using nutrient agar (Difco, 1.5%) enriched with 2% glycerol; the agar overlay was prepared from nutrient broth (Difco) by the addition of 0.7% Bacto-agar (Difco). Phage stocks were maintained and phage dilutions were made in HIB (Difco) except for the phage stock used for antiserum preparation where physiological saline, 0.85% NaCl, was used. All phage and bacterial cultures were stored at 4 C.

The selective medium used to isolate xyl^+ transductants initially consisted of the following ingredients: K_2HPO_4, 1.5 g; KH_2PO_4, 0.5g; NH_4Cl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; Na_2SO_4, 0.2 g; purified agar (Difco), 17.0 g in the basal layer, 8.0 g in the top layer; D-xylose (Pfanstiehl, A grade, chromatographically homogeneous, Waukegan, Illinois), 10.0 g; 0.4% bromcresol purple (National Aniline, New York, New York), 75 ml; H_2O to 1 liter. All ingredients were sterilized by autoclaving except for the xylose. This was sterilized by membrane filtration (through a 0.45μ filter; Millipore Corp., Bedford, Massachusetts). This medium which will be hereafter referred to as MM was recommended by Bönicke and was his modification of a medium used by Gordon for carbohydrate utilization studies (Gordon and Mihm, 1959). Later modification of this medium consisted of the following: 1) $FeCl_3$, 0.05 g; and 2) streptomycin sulfate (Sigma, St. Louis, Missouri), 0.05 g, were added; and 3) bromcresol purple was eliminated. The medium with all three modifications will be hereafter referred to as MM'.

The media used to isolate drug resistant transductants consisted of either 1) nutrient agar (Difco, 1.5%) basal agar; first semi-solid agar layer

prepared from nutrient broth (Difco) by the addition of 0.7% Bacto-agar (Difco); second semi-solid agar layer prepared as the first but with 100 µg/ml of the appropriate drug, or 2) MM' plus 100 µg/ml of the appropriate drug in the top layer. Drugs employed in these studies were streptomycin sulfate (Sigma), D-cycloserine (Sigma), ethambutol (Myambutol; Lederle Corp., a subsidiary of American Cyanamid, Pearl River, New York), rifampicin (Rifadin; Dow Chemical Company, Zionsville, Indiana), and isonicotinic acid hydrazide (INH; Sigma).

The selective media used to isolate auxotrophic mutants consisted of MM' with and without agar, with sodium citrate (0.1% w/v) and 2% glycerol (2% v/v) in place of D-xylose as the carbon sources. These were the carbon sources used by Juhasz (1968) and by Ratledge and Hall (1970) and by Holland and Ratledge (1971) in previous studies of mycobacterial auxotrophs.

D. *Primary characterization of bacterial strains.* Two major tests were employed: Bönicke's amidase test (Bönicke, 1960) and Bönicke and Kazda's carbohydrate-nitrite test (Bönicke and Kazda, 1970). These were used to identify strains during all phases of this study.

1. *Preparation of suspensions for enzymatic studies.* All strains of *M. phlei* and lysogens (obtained from S.E. Juhasz) were heavily inoculated on Lowenstein-Jensen plates and incubated for 8-10 days at 37 C. The growth was scraped off the surface using a sterile scalpel blade and suspended in a tared tube containing physiological saline. The bacteria were washed three times in physiological saline and resuspended in the appropriate buffer, pH 7.2 M/15 sodium-potassium phosphate for the amide series, or pH 7.0 M/15 sodium-potassium phosphate containing 20 µg $NaNO_2$ per ml for the carbohydrate nitrite

series, to a final concentration of 10 mg moist weight per ml.

2. *Amidase test.* Bönicke's amide series was employed to test for amidase activity. A 0.5 ml buffered suspension of a given strain was mixed with 0.5 ml of 0.00164 M solution of each of the following amides: acetamide, benzamide, urea, isonicotinamide, nicotinamide, salicylamide, pyrazinamide, succinamide, malonamide, and allantoin. These were sterilized by heating at 100 C in a water bath for 30 min, except for urea and allantoin which were filtered through a 0.45 μ membrane filter (Millipore). Each amide-bacterium mixture was incubated for 22 hr at 37 C. Liberation of ammonia resulting from the hydrolysis of a given amide was detected by the phenol-hypochlorite method described by Russell (1944). One tenth ml of 0.003 M $MnSO_4 \cdot 6H_2O$, 1.0 ml of phenolate reagent, and 0.5 ml of KClO was added to each tube, in the given order, with a minimum delay between the phenolate and the hypochlorite. Since this method, originally reported by Berthelot (1859) does not produce a stable colored complex, time is critical when determining the concentration of the complex (Weatherburn, 1967). Color development was obtained by heating the tubes in water bath at 100 C for 15 min. Five min. were allowed for cooling at room temperature. The tubes were then read visually as positive if blue, and negative if yellow-green.

Reagents for amidase test and their preparation. The phenolate was prepared by suspending 25 g of phenol (reagent grade crystal) in 10 ml distilled water, adding 54 ml of % N NaOH and adjusting the solution to a final volume of 100.0 ml. This solution had to be freshly prepared. The hypochlorite (Eau d'Javel, Germany) was provided by Bönicke. Benzamide, nicotinamide, salicylamide, succinamide and malonamide were obtained from

J. T. Baker Chemicals, Phillipsburg, New Jersey; acetamide, urea, and isonicotinamide, from Sigma; allantoin and pyrazinamide were provided by Bönicke. The former was a product of Fluka A. G., Buchs, S. G., Switzerland, and the latter of Krugman and Company, Hamburg, Germany. Other chemicals were analytical reagent grade.

3. *Carbohydrate-nitrite test.* The recently developed carbohydrate-nitrite series of Bönicke and Kazda was used to test which carbohydrates could serve as electron donors to reduce nitrite (Bönicke and Kazda, 1970). A 0.5 ml of the buffered suspension of each strain was mixed with 0.5 ml aqueous solutions containing 40 µg/ml of one of the following carbohydrates: glucose, rhamnose, xylose, arabinose, mannose, galactose, mannitol, sorbitol, inositol, dulcitol, lactose, raffinose, maltose, trehalose, levulose, sucrose and one solution containing 1.0% glycerol. These tubes were then incubated for 6 days at 37 C. After incubation, 0.1 ml of a 1:1 dilution of concentrated HCl was added to each tube and mixed on a vortex mixer for 15-30 sec. A 0.1 ml of aqueous 0.2% sulfanilamide and 0.1 ml of aqueous 0.01% N-1-naphthylethylene-diamine were then added to each tube. Color developed after approximately 5 min. at room temperature. The color reaction is based on the formation of a diazocomplex. This involves first the reaction in acid solution of a primary amine, such as sulfanilic acid or sulfanilamide with nitrite to form a diazonium salt. The latter is then coupled to an aromatic amine to yield the red azo dye (Shinn, 1941). In this test, tubes which produce the red complex are negative since nitrite is still present and tubes that produce no color are positive since this indicates the nitrite has been reduced. This is a semiquantitative test with tubes read simply as positive or negative if nitrite

is either absent or present, respectively.

Reagents for carbohydrate-nitrite test. Sulfanilamide and glycerol were obtained from Eastman Organic Chemicals, Rochester, New York; N-1-naphthylethylenediamine from Mann Research Laboratories, New York, New York. The carbohydrates were all obtained from Pfanstiehl. Other chemicals were analytical reagent grade.

E. *Secondary bacterial characterization.* Once the donor and recipient strains were selected, further biochemical and other taxonomic tests were employed to determine the relationship of the two strains to each other.

1. *Acid fast staining technique.* All strains during all phases of the study were first identified as mycobacteria by a positive acid fast test employing either the Ziehl-Neelsen stain or the Acid Fast Rapid Staining technique (Gugol Corp., North Elmsford, New York). Carbol fuchsin and Loeffler's methylene blue were obtained from Fischer Scientific, Fair Lawn, New Jersey.

2. *Growth characteristics.* Growth at 37 C, 45 C and 52 C was tested by inoculating Löwenstein-Jensen medium slants with approximately 10^6 colony forming units (CFU) of the strain and incubating them at the specified temperature until growth occurred or for 14 days. Survival at 60 C was tested by incubating a saline suspension containing about 10^6CFU of the strain sealed in a glass ampule, in a water bath set at 60 ± 0.5 C for 60 min. The ampule was then opened and the contents were diluted $1:10^2$ and $1:10^4$. The undiluted contents as well as the dilutions were inoculated onto Löwenstein-Jensen slants which were incubated at 37 C until growth occurred. An identical ampule containing the same saline suspension not incubated at

60 C was used as a control. If reduction in population was less than 10% the strain was considered as able to survive heating to 60 C for 1 hr. Pigmentation was observed on Löwenstein-Jensen slants and plates incubated at 37 C. No attempt to extract pigment was made.

3. *Phage susceptibility.* The strains were tested for susceptibility to the two phages available which could lyse only one species and thus were suitable for phage typing (Bönicke and Juhasz, 1965), smegmatis phage Bo1 and phlei phage Bo2 each previously propagated on their original host *M. smegmatis* SN2 for the former and *M. phlei* F89 for the latter. An aliquot of 10^6 plaque forming units (PFU) of each phage was dropped onto a lawn comprised of the strain to be tested. The plates were incubated at 37 C for 5 days. Confluent lysis was considered as positive.

4. *Drug resistance pattern.* Resistance to 1, 10, and 100 µg/ml of the five commonly employed anti-mycobacterial drugs, streptomycin, isonicotinic acid hydrazide, D-cycloserine, ethambutol, and rifampicin were recorded. Drugs were incorporated into nutrient agar slants and 10^6 CFU of each strain was inoculated. The tubes were incubated at 37 C for 14 days. Slants containing no drug were used as controls. Slants with drugs that produced growth approximately equal to that of the control were considered as positive.

5. *Additional biochemical tests.* The sugar test of Gordon (Gordon and Mihm, 1959) was used to test for ability of a strain to produce acid from a given carbohydrate within 28 days. The modification of their medium described earlier (MM) containing the appropriate carbohydrates was employed. All of the carbohydrates studied in the carbohydrate-nitrite series were also studied in the sugar test. Change in pH suitable to convert the bromcresol purple to

its yellow form was considered positive. Upon selection of suitable carbohydrates, the strains were tested for their ability to utilize these carbohydrates as a sole carbon source using the same medium as in the sugar test but considering any growth within 28 days as positive. The nitrate reduction test of Virtanen (1960) as modified by Juhasz et al. (1969), was used to test for the ability of the strain to reduce nitrate to nitrite. A 0.04 ml sample of a suspension containing 0.04 mg moist weight of bacteria was inoculated into nitrate broth (Difco) and incubated at 37 C for 4 hr. The tubes were developed as described for the carbohydrate-nitrite test, but for this test formation of the red complex was considered positive. Wayne's modification (Wayne, 1961) of the arylsulfatase test of Whitehead, Wildy and Engbaek (1953) was employed to test for the ability of a strain to hydrolyse arylsulfates. Dubos' oleic acid agar (Difco) containing 650 mg of phenolphthalein disulfate tripotassium salt (Eastman) per liter was distributed in 2.0 ml amounts into tubes. Tubes were inoculated with 0.4 ml moist weight of bacteria and incubated for 3 and 14 days. Any phenolphthalein liberated by hydrolysis of the substrate was detected by the addition of 0.2 ml of 2N Na_2 CO_3. The tubes were kept at 4 C to permit diffusion of the color through the medium (Juhlin, 1967) and then visually compared to the control. *M. fortuitum* strain SN203 from the Borstel Collection was used as the positive control. Tubes showing approximately the same color as the positive control were considered positive.

6. *Bacterial growth rate studies.* Studies of bacterial growth in standing cultures was made of the recipient and donor strains SN109 and F89 as well as for *M. smegmatis SN2*. Cultures containing approximately 10^8CFU/ml,

kept at 4 C for 48 hr were diluted 1:100 into fresh sterile HIB to give a final volume of 100 ml. The containers used for these experiments were 250 ml screw-capped Erlenmeyer flasks. The flasks were incubated at 37 C. Samples were taken at 8 hr intervals over a 96 hr period. The samples were diluted in HIB and assayed for viable count using the agar overlay method. A 0.1 sample was added to 3 ml of melted but cooled soft agar and poured over the basal layer. All assays were performed in duplicate.

F. *Purification of bacterial strains used for transduction experiments.* Strains SN109 and F89 selected for transduction experiments, were purified by six serial single colony transfers on nutrient agar. In addition lysogens employed for high frequency transduction were passed three times in HIB containing 0.05% Tween-80 (Eastman, Rochester, New York) after purification on nutrient agar.

G. *Purification of phage stock.* Three single-plaque transfers of phage Bo2 were made. First, the phage suspension was serially diluted to single plaques. A well isolated typical plaque was picked with a sterile inoculating loop and suspended in 1.0 ml sterile HIB. This was again diluted to single plaques and the process was repeated three times.

H. *Phage propagation and counting of phage particles.* Phage was assayed by the plaque count method introduced by Gratia (Adams, 1959), as modified by Froman, et al., (1954) for use with mycobacteriophages. Indicator strain SN109 or F89 organisms were inoculated into melted but cooled to 52 C semi-solid agar and poured onto a nutrient agar base plate. After solidification of the top agar layer, 0.05 ml of ten-fold serial dilutions of the phage were dropped in duplicate, one sample on each half of a plate. After 5 days

incubation at 37 C, the plates were assayed. The titer was determined as the number of individual plaques multiplied by the dilution and extrapolated for 1.0 ml of the undiluted phage suspension.

I. *Selection of drug resistant bacterial mutants.* Bacterial strains were propagated on nutrient agar slants containing halving serial dilutions of the appropriate drug with the highest concentration being 1 mg/ml and the lowest concentration being approximately 0.1 µg/ml. Colonies found on slants containing fewer than 50 colonies were transferred to slants of higher drug concentration and the process repeated until a mutant which could tolerate >125 µg/ml was isolated. These strains were then plated on Löwenstein-Jensen medium and nutrient agar and then replated onto plates containing 100 µg/ml of the appropriate drug. Mutants thus selected were purified as described earlier.

J. *Preparation and selection of auxotrophic mutants.* The nitrosoguanidine method of Adelberg, Mandel and Chen (1965), as modified for mycobacteria by Holland and Ratledge (1971), was employed to obtain auxotrophic mutants of *M. phlei*.

1. *Nitrosoguanidine toxicity studies on M. phlei.* *M. phlei* SN109 was incubated at 37 C for 48 hr, centrifuged, washed with sterile physiological saline and resuspended in minimal medium broth to a concentration of approximately 10^8 colony forming units (CFU) per ml with nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine, Aldrich Chemical Company, Milwaukee, Wisconsin) at various concentrations. This was incubated at 37 C for specified periods of time. After incubation viable counts were made on nutrient agar plates.

2. *Mutagenesis.* Strain SN109 incubated for 48 hr at 37 C, washed and resuspended in minimal medium to a concentration of approximately 10^8 CFU per ml was exposed to nitrosoguanidine at that concentration and time that would kill greater than 90% but less than 99.9% of the population. After incubation samples were collected on membrane filters and the bacteria were washed with 30 ml of sterile physiological saline and resuspended in nutrient broth and reincubated for 24 hr at 37 C to allow for phenotypic expression of mutants. The suspensions were then collected on membrane filters and washed with 30 ml of saline.

3. *Enrichment of mutants.* The samples on the membrane filters were then resuspended in a minimal medium containing 1 mg isonicotinic acid hydrazide (INH) per ml and incubated for an additional 24 hr at 37 C. Following this incubation the bacteria were again washed with physiological saline, resuspended in nutrient broth and serially diluted and plated onto nutrient agar to obtain a master plate containing 50-150 colonies.

4. *Selection of mutants.* The master plates were replica plated according to the method of Lederberg and Lederberg (1952) onto plates containing minimal medium, minimal medium plus casamino acids (Difco, vitamin free) 20 mg/ml, minimal medium plus purines and pyrimidines (Sigma, nucleotide kit) 20 µg/ml each, minimal medium plus vitamins (Sigma, vitamin kit) 1 µg/ml each, and onto minimal medium plus yeast extract (Difco, 1%). Colonies found on the plates supplemented with yeast extract and on plates with either vitamins, nucleotides, or amino acids, but not on unsupplemented minimal medium were transferred onto media containing specific vitamins, nucleotides, or amino acids to determine their specific nutritional

requirements. The individual L-amino acids were obtained from Sigma.

K. *Preparation of transducing phage stocks.* Phage Bo2 was lytically propagated on the donor strain SN109 which can utilize xylose as a sole carbon source (xyl^+) and is sensitive to 10 µg/ml streptomycin (str-s). The zone of lysis was removed from these plates after 4-5 days and resuspended in HIB at a concentration of 5-7 plates of lysis per 5.0 ml HIB. This was mixed on a vortex mixer (Genie; Scientific Products) for 3-5 min and then centrifuged at low speed to sediment agar. The supernate was then membrane filtered into a sterile screw-capped tube. This stock culture was assayed for phage titer and tested for sterility both by streaking nutrient agar plates and inoculating sterile HIB. Both were incubated at 37 C for 4 weeks or until growth had occurred. To eliminate transforming DNA these preparations were incubated with DNase at a final concentration of 25 µg/ml at 37 C for 30 min. To obtain HFT lysates, transductants shown to be lysogenic were grown in HIB for 8-10 days. Bacteria were then removed by membrane filtration.

L. *Preparation of recipient bacterial cultures.* Strain F89 was chosen as recipient because this organism cannot utilize xylose as a sole carbon source (xyl^-). For all transduction experiments except the first, a mutant of this strain, resistant to over 50 µg/ml streptomycin (str-r) was employed. It was propagated on Löwenstein-Jensen plates for 7-10 days. The growth was scraped off using a sterile scalpel blade being careful not to include any medium in the scrapings. The scrapings were suspended in HIB to a final concentration of 10.0 mg wet weight of bacteria per ml. The contents of these tubes were assayed by viable counts on nutrient agar using the agar overlay method. During the 8-10 days which were required to obtain viable counts the HIB

suspensions were kept at 4 C.

M. *Procedure of transduction experiments.* The stock recipient bacterial culture was diluted to a concentration of approximately 10^8 CFU per ml. This was reincubated as standing culture for specified periods at 37 C. A 1.5 ml aliquot of bacterial suspension then was mixed with an equal volume of phage Bo2 propagated on the donor strain. The phage suspension in later experiments had been preincubated with DNase (B grade; Calbiochem, Los Angeles, California; 30 µg/ml) for 30 min at 37 C. The concentration of phage was between 10^8 - 10^9 PFU per ml. The mixture of phage and bacteria was incubated as a standing culture at 37 C for specified periods of time. The exact ratio of phage to bacterium, the multiplicity of infection (MOI), varied in different experiments. Viable counts were made prior to and following incubation with phage. The incubation mixtures were collected on membrane filters, washed in 30 ml of physiological saline, and resuspended in 3.0 ml of physiological saline. This suspension was then plated out onto selective medium using the agar overlay technique. In the case of drug resistant transfers the bacteria were not washed with saline and a second overlay containing the drug was added after specified periods of time 0, 6, 24 and 48 hr. These plates were then incubated at 37 C and scored after 4 weeks.

N. *Controls used in transduction experiments.* The controls employed in these experiments consisted of recipient bacterial cultures incubated with phage (Bo2·F89) not propagated previously on the donor bacterium, with phage plus DNase, with phage plus antiphage serum, with heat killed phage (boiled for 30 min at 99.5 ± .5 C or autoclaved at 120 C for 15 min) or with sterile HIB in place of phage. Samples of controls were processed exactly the same

as samples from the experimental tubes.

O. *Production of antiphage serum.* A 1 ml aliquot of phage Bo2 suspended in sterile physiological saline (0.85% NaCl) was injected into two male albino rabbits twice weekly, once intravenously and once intraperitoneally each week, for five weeks. The concentration of phage was about 10^9 plaque forming units per ml. An identical rabbit was injected with sterile physiological saline without phage at the same time as the other two. After a one week rest period, blood samples from the rabbits injected with phage were taken from the ear vein. Upon determination that antiphage serum could neutralize the entire phage population when 10^6 PFU/ml was used as a test concentration, all of the animals were bled by cardiac puncture. Blood samples were collected in 20 ml sterile screw-capped tubes and allowed to clot at room temperature for 30 min. The clot was removed from the sides of the tubes with a sterile glass rod and the tubes were placed at 4 C overnight to allow serum and cells to separate. The tubes were then centrifuged, and the supernatant serum was carefully removed from the sedimented blood cells, placed in screw-capped tubes (5 ml per tube) and stored at -4 C. The antiphage serum was employed as a control with phage in transduction experiments and was also employed as a method of eliminating unadsorbed phage both in the one step growth studies and single burst experiments.

P. *Neutralization test procedures.* Neutralization tests were performed according to the methods described by Adams (1959). The stock phage suspension was diluted in HIB to a concentration of about 1 x 10^6. The rabbit serum was first heated to 56 C for 30 min to inactivate complement, allowed to cool and then tested at undiluted strength and at dilutions of 1/10 and

1/100 in HIB. A 0.1 ml sample of the diluted prewarmed (37 C) phage preparation was added to 0.9 ml of prewarmed antiserum and incubated at 37 C. At specified time intervals, the number of free phage was determined by diluting the reaction mixture 1:100 with HIB and plating 0.1 ml of this upon a bacterial indicator background of strain SN109. This resulted in about 100 PFU/plate in tubes containing no antiserum. The amount of neutralization was determined by comparing the number of plaques formed after exposure of the phage to antiserum with the number of plaques formed in the controls. Two controls were used: one in which sterile HIB was substituted for antiserum; and one in which serum from the rabbit injected with saline without phage was used in place of the antiphage serum. All tests were done in duplicate.

Q. *Characterization of bacteriophage Bo2·SN109 propagated on strain SN109.* To better understand the transduction system it was necessary to know more about the phage.

1. *Plaque morphology studies.* Plaque morphology studies were made on Bordet Gengou agar (Difco) enriched with 2% glycerol using *M. phlei* SN109 as the background bacterial strain. This strain was employed as the propagating host of phage used for all of the following studies:

2. *Preparation of specimens for the electron microscopic morphological examination.* A zone of confluent lysis was obtained by dropping a few drops of the concentrated phage stock upon a background of bacterial indicator. The Petri dish was inverted and 3 ml of a 2% solution of OsO_4 was put on a piece of filter paper which had been placed in the cover. For 2 hr, the vapors arising from this cover were allowed to fix the phage in the zone of

confluent lysis just above it. At the end of this time, the Petri dish was again placed right side up and a small volume of phage was washed from the surface of the plate with distilled water. A drop of this was placed on a 300 mesh copper grid, which had previously been coated with 0.5% formvar. After the drop dried, the phage on the grid was stained with 0.2% uranyl acetate for 10 seconds and allowed to dry. The specimens were then viewed on a Hitachi Perkin-Elmer HU-11A electron microscope.

3. *Isolation of bacteriophage DNA.* DNA was isolated from phage Bo2 by the cold phenol method of Marmur (1961). A 5 ml phage suspension containing 6×10^9 PFU/ml was treated with equal volumes of water-saturated phenol (spectrophotometric grade; Allied Chemical Company, Morristown, New Jersey). The tube was inverted 10 times and placed in an ice water bath for 10 min. This was repeated 3 times. Denatured protein was sedimented by centrifuging at 2,000 xg for 15 min. The top aqueous layer was carefully removed with a wide-bore pipet and transferred to a sterile screw-capped tube. This was dialized against 2 liters of saline citrate buffer (SSC) for 24 hr to remove the extracting solvent. SSC consists of 0.15M sodium chloride and 0.015M sodium citrate. The content of the dialysis tubing was transferred to another sterile screw-capped tube, in which the DNA was precipitated with 2 volumes of cold isopropanol. This was centrifuged and the supernatant was drawn off, leaving the precipitated DNA which was resuspended in SSC and refrigerated until used.

4. *Isolation of bacterial DNA.* To compare the guanine+cytosine (GC) ratio of the phage to its host, it was necessary to obtain bacterial DNA from the two *M. phlei* strains SN109 and F89 and also from *M. smegmatis* strains

SN2 and SN46 for comparison.

Mycobacterial cells taken from Löwenstein-Jensen plates with a scalpel were washed with distilled water, centrifuged, and collected in 1:10 dilution of SSC in distilled water to a final concentration of 1 gm/ml. An equal weight of glass beads was added to this suspension, which was poured into a disintegrator bottle and cooled in an ice water bath. The cells were then broken for 15 seconds in a cell disintegrator (Braun; Quigley-Rochester, Inc., Rochester, New York) operating at 4,000 cycles per second. Unbroken cells, glass beads, and larger cell debris were removed by centrifugation at 3,000 xg for 10 min, and the supernatant fraction was carefully pipetted off and placed in a sterile screw-capped tube. Just as for the DNA extracted from the phage, equal volumes of H_2O saturated phenol were added to this supernatant fraction. The contents of the tube were gently rocked back and forth 10 times, and placed in an ice water bath. This was allowed to stand for 20 min, and the procedure was repeated twice. The aqueous layer was removed after centrifugation at 2,000 xg for 10 min, and the DNA was precipitated by 2 volumes of cold isopropanol, and collected by centrifugation. This was then dissolved in SSC buffer and treated with pancreatic RNase (Calbiochem) which was boiled for 15 min to remove any trace of contaminating DNase. The RNase treatment was done at a concentration of 50 µg/ml at 37 C for 1 hr. The DNA was again precipitated with 2 volumes of cold isopropanol, centrifuged, the supernatant removed, and the DNA was resuspended in SSC. Finally, this was dialyzed against 2 changes of 2 liters of SSC for 38 hr. The dialysate was removed with a wide-bore pipette, put into a sterile screw-capped tube, and stored in the refrigerator at 4 C.

5. *Determination of buoyant density by cesium chloride density gradient centrifugation.* Buoyant densities of bacterial and phage DNA preparations were determined by centrifugation at 44,770 rev/min at 25 C in CsCl as described by Schildkraut et al. (1962) with an analytical ultracentrifuge (Beckman, Spinco model E, with an AN-D rotor). The DNA of *Micrococcus lysodeikticus* (ρ = 1.7310) kindly provided by L. DeSalle was used as a known marker. Also, DNA of *Mycobacterium smegmatis* strain SN46 was used as an additional marker. Pictures of the DNA bands formed were taken after 24 hr of centrifugation, using ultra-violet absorption photography. Tracings were made of the film containing the bands of DNA with a densitometer. The difference between the distance that the unknown DNA bands moved and the distance that the marker DNA moved was computed, and the buoyant density was calculated using the formula of Schildkraut, et al., (1962)

$$\rho = \rho_o + 4.2\omega^2 (r^2 - r_o^2) \text{ g cm}^{-3}$$

At 44,770 rev/min this formula converts to:

$$\rho = \rho_o + 0.0092 (r^2 - r_o^2) \text{ g cm}^{-3}$$

where ρ = density of the unknown DNA

ρ_o = the density of the standard DNA

r_o = the distance of the standard DNA from the center of rotation

r = the distance of the unknown DNA from the center of rotation

To determine the buoyant density, the following formula was used:

$$\rho = 0.098 (GC) + 1.660 \text{ g cm}^{-3}$$

$$\text{or } GC = \frac{\rho - 1.66 \text{ g cm}^{-3}}{0.098}$$

where ρ = buoyant density in g cm^{-3}

and GC = mole fraction guanine plus cytosine

Samples were treated with DNase and then analysed on the analytical ultracentrifuge to prove that what was being measured was indeed a band of DNA. This was done even though the banding and absorption characteristics were quite typical of DNA.

A 12 mm standard cell (which held 0.83 ml of the CsCl-DNA solution in the rotor) was used. Enough CsCl was added to bring the mean density of the DNA solution to 1.710 gm cm^{-3}. Also, the concentration of DNA was adjusted to between 3 and 10 µg/ml, using the Molar Extinction Coefficient of DNA at 260 nm of 20 (i.e., 20 OD_{260} units = 1000 µg/ml DNA). This concentration of DNA was used in the cell because it resulted in a band that was sharp and not too wide.

6. *Procedure for adsorption experiments.* The method followed was a modification of that described in Adams (1959). Host bacteria were grown on Löwenstein-Jensen plates and dense suspensions were prepared in screw-capped tubes containing 10 ml sterile HIB. This stock was placed in the refrigerator after titration, to be used for both the adsorption and the single-step growth studies. At the time of testing, 0.1 ml of refrigerated stock was added to 0.9 ml of HIB and pre-incubated for 24 hr to bring the culture into early logarithmic phase. After pre-incubation the phage was added to the bacteria at an MOI of 0.001. At 10 min intervals for 1 hr, 0.1 ml of the incubation mixture was withdrawn and placed into 9.9 ml HIB in order to dilute the mixture and stop adsorption. This dilution was centrifuged on a size 2, International Equipment Company centrifuge at 1,000 xg for 10 min. A 0.2 ml sample was carefully withdrawn from the top, and 0.1 ml of undiluted and 1:10 diluted suspension was placed upon the background

indicator strain. One control was the sample withdrawn and centrifuged immediately, after adding the phage to the bacteria. Another control involved adding the phage to HIB without bacteria, and centrifuging and titrating this system. The percent of phage remaining unadsorbed was calculated as the number of phage/ml in the supernatant at t_n over the number of phage/ml at t_o (control) x 100. Percent adsorption was then computed by subtracting the percent remaining unadsorbed from 100 percent.

7. *Procedure for the single-step growth experiments.* The method followed was largely that described in Adams (1959). Host bacterial suspensions were prepared as described above in the adsorption procedure. Adsorption was allowed to take place at an MOI of 0.001, since adsorption of at least 50% could only be obtained at this low multiplicity. After 24 hr or 48 hr pre-incubation of 9 ml of host cells (2-6 x 10^9 CFU/ml) suspended in HIB, 1.0 ml of phage (1-4 x 10^7 PFU/ml) was added and adsorption begun. Adsorption was allowed to proceed for 30 min, and then 0.1 ml was withdrawn from the tube and added to 0.9 ml of the appropriately diluted homologous complement-inactivated antiphage serum. Antiserum with over 95% neutralization capacity was used in all cases. After 30 min of exposure to the antiserum, a 1:100 dilution was made in HIB. Samples of 0.1 ml were withdrawn at 20 min intervals for the first 2 hr, and at 30 min intervals for the remaining 7.5 hr, and placed on a background of host bacteria. All tests were done in duplicate.

8. *Procedure for single burst experiments.* The method followed was largely that described in Adams (1959). Host bacterial suspensions were prepared as described above in the adsorption procedure. Adsorption was allowed to take place at an MOI of about 40. After 24 hr pre-incubation of

9 ml of host cells (2-6 x 10^7 CFU/ml) suspended in HIB, 1.0 ml of phage (4 x 10^8 PFU/ml) was added and adsorption begun. Adsorption was allowed to proceed for 30 min, and then 0.1 ml was withdrawn from the tube and added to 0.9 ml of the appropriately diluted homologous complement-inactivated antiphage serum. Antiserum with over 95% neutralization capacity was used in all cases. After 30 min of exposure to the antiserum a 1:10^4 dilution was made in HIB and 0.3 ml of this dilution was then added to 41.7 ml HIB to give a final concentration of about 0.7 CFU/ml. This was then distributed in 0.5 ml aliquots to 50 sterile tubes. The tubes were incubated at 37 C for an additional 7 hr making the overall incubation period 8 hr. After this incubation 3.5 ml of top agar (Difco, Nutrient Agar 1.5% in an equal volume of Difco, Nutrient broth) containing *M. phlei* SN109 at greater than 10^9 colony forming units per ml was added to each tube and the contents were poured over a basal agar plate and incubated at 37 C. These were added to top agar containing SN109 as had been done with the separate samples and poured over basal agar plates which were then incubated at 37 C. The results of all plates were tabulated by the method described in Adams (1959).

A. *Primary characterization of Mycobacterium phlei strains.*

1. *Amidase test.* The results of the Bönicke amidase test for all strains of *M. phlei* and their lysogens (Table 1) show that all strains were capable of hydrolysing acetamide, urea, nicotinamide and pyrazinamide but not benzamide, isonicotinamide, salicylamide, allantoin, succinamide, or malonamide after 22 hr incubation. With regard to this amide series, there were no significant differences between any of the strains including the lysogens.

2. *Carbohydrate-nitrite test.* The results of the Bönicke-Kazda carbohydrate-nitrite test (Table 2) demonstrate that strain F89 and its lysogens can utilize glucose, arabinose, mannose, galactose, mannitol, sorbitol, trehalose, fructose and glycerol as suitable electron donors for the reduction of nitrite but could not utilize rhamnose, inositol, dulcitol, lactose, raffinose, maltose, sucrose and, most importantly, xylose as electron donors. All other strains of *M. phlei*, including SN109 and its lysogens, could utilize glucose, arabinose, mannose, galactose, mannitol, sorbitol, trehalose, fructose, glycerol, as well as xylose but not rhamnose, inositol, dulcitol, lactose, raffinose, maltose and sucrose as suitable electron donors for the reduction of nitrite. In summary, the only difference between F89, its lysogens and all other *M. phlei* strains tested in these experiments, was the inability of the former to utilize xylose as an electron donor to reduce nitrite. This provided the possible marker sought.

TABLE 1. Amidase activity of *M. phlei* strains after 22 hr.

Strain	Acetamidase	Benzamidase	Urease	Isonicotinamidase	Nicotinamidase	Pyrazinamidase	Salycilamidase	Allantoinase	Succinamidase	Malonamidase	Control (.85% NaCl)
SN101	+	-	+	-	+	+	-	-	-	-	-
SN102	+	-	+	-	+	+	-	-	-	-	-
SN103	+	-	+	-	+	+	-	-	-	-	-
SN104	+	-	+	-	+	+	-	-	-	-	-
SN105	+	-	+	-	+	+	-	-	-	-	-
SN106	+	-	+	-	+	+	-	-	-	-	-
SN107	+	-	+	-	+	+	-	-	-	-	-
SN108	+	-	+	-	+	+	-	-	-	-	-
SN109	+	-	+	-	+	+	-	-	-	-	-
SN109 (Bo2·F89)	+	-	+	-	+	+	-	-	-	-	-
SN110	+	-	+	-	+	+	-	-	-	-	-
SN111	+	-	+	-	+	+	-	-	-	-	-
SN112	+	-	+	-	+	+	-	-	-	-	-
SN113	+	-	+	-	+	+	-	-	-	-	-
SN114	+	-	+	-	+	+	-	-	-	-	-
SN115	+	-	+	-	+	+	-	-	-	-	-
SN118	+	-	+	-	+	+	-	-	-	-	-
SN119	+	-	+	-	+	+	-	-	-	-	-
F89	+	-	+	-	+	+	-	-	-	-	-
F89 (Bo2·F89)	+	-	+	-	+	+	-	-	-	-	-
F89 (Bo2·SN109)	+	-	+	-	+	+	-	-	-	-	-

TABLE 2. Carbohydrate nitrite tests of *M. phlei* strains after 6 days.

Strain	Glucose	Rhamnose	Xylose	Arabinose	Mannose	Galactose	Mannitol	Sorbitol	Inositol	Dulcitol	Lactose	Raffinose	Maltose	Trehalose	Fructose	Sucrose	Glycerol	Control (No Carbohydrate)
SN101	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN102	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN103	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN104	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN105	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN106	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN107	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN108	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN109	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN109 (Bo2·F89)	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN110	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN111	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN112	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN113	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN114	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN115	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN118	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
SN119	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
F89	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
F89 (Bo2·F89)	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
F89 (Bo2·SN109)	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-

B. *Further characterization of M. phlei recipient and donor strains.*

Strain F89 was established as the suitable recipient strain for genetic transfer experiments. Strain SN109 was randomly selected as donor from the *M. phlei* strains which could utilize xylose. The results of the growth characteristic studies, phage susceptibility tests, drug resistance patterns and additional biochemical tests show that strains F89 and SN109 gave similar reactions (Tables 3a and 3b). Both strains grew at 37 C, 45 C, 52 C and survived at 60 C for 4 hr. The colonial morphology and pigmentation were quite similar in the two strains. Both strains were susceptible to phage Bo2 but resistant to phage Bol. Both strains were resistant to 1 μg/ml but susceptible to 10 and 100 μg/ml streptomycin. They were resistant to 1, 10, and 100 μg/ml of isonicotinic acid hydrazide (INH) and to 1 and 10 μg/ml D-cycloserine but susceptible to 100 μg/ml of the latter. Both strains were resistant to 1 and 10 μg/ml ethambutol, although SN109 showed decreased growth at 10 μg/ml, and susceptible to 100 μg/ml. They were equally susceptible to 0.1, 1.0, and 10 μg/ml rifampicin. Either strain reduced nitrate to nitrite and was arylsulfatase positive after 14 days but not after 3 days. Both strains produced acid within 28 days from glucose, arabinose, mannose, galactose, mannitol, sorbitol, trehalose and fructose, but not from rhamnose, inositol, dulcitol, lactose, raffinose, maltose, sucrose or glycerol. Both strains utilized glucose, arabinose, mannose, galactose, mannitol, sorbitol, trehalose, fructose and glycerol as a sole carbon source but can not utilize rhamnose, inositol, dulcitol, lactose, raffinose, maltose or sucrose. As mentioned earlier the one major difference was that SN109 could utilize xylose as a sole carbon source while F89 could not.

TABLE 3a. Comparison of *M. phlei* strain F89 and SN109.

Test	Strain SN109	Strain F89
Growth at 45 C	+	+
Growth at 52 C	+	+
Survival at 60 C for 4 hr	+	+
Pigmentation on nutrient agar	+	+
Susceptibility to phage Bo1	-	-
Susceptibility to phage Bo2	+	+
Resistance to streptomycin 1 µg/ml	+	+
Resistance to streptomycin 10 µg/ml	-	-
Resistance to streptomycin 100 µg/ml	-	-
Resistance to INH 1 µg/ml	+	+
Resistance to INH 10 µg/ml	+	+
Resistance to INH 100 µg/ml	+	+
Resistance to D-cycloserine 1 µg/ml	+	+
Resistance to D-cycloserine 10 µg/ml	+	+
Resistance to D-cycloserine 100 µg/ml	-	-
Resistance to ethambutol 1 µg/ml	+	+
Resistance to ethambutol 10 µg/ml	+	+
Resistance to ethambutol 100 µg/ml	-	-
Resistance to rifampicin 0.1 µg/ml	-	-
Resistance to rifampicin 1.0 µg/ml	-	-
Resistance to rifampicin 10 µg/ml	-	-
Nitrate reduction (4 hr)	+	+
Arylsulfatase production (3 day)	-	-
Arysulfatase production (14 day)	+	+

TABLE 3b. Comparison of *M. phlei* strain F89 and SN109.

Test	Strain SN109	Strain F89
Acid production from carbohydrates		
glucose	+	+
rhamnose	-	-
xylose	+	-
arabinose	+	+
mannose	+	+
galactose	+	+
mannitol	+	+
sorbitol	+	+
inositol	-	-
dulcitol	-	-
lactose	-	-
raffinose	-	-
maltose	-	-
trehalose	+	+
fructose	+	+
sucrose	-	-
glycerol	-	-
Utilization of carbohydrate as electron donor		
glucose	+	+
rhamnose	-	-
xylose	+	-
arabinose	+	+
mannose	+	+
galactose	+	+
mannitol	+	+
sorbitol	+	+
inositol	-	-
dulcitol	-	-
lactose	-	-
raffinose	-	-
maltose	-	-
trehalose	+	+
fructose	+	+
sucrose	-	-
glycerol	+	+

C. *Choice of selective medium and other preliminary experiments.*

1. *Selective medium.* Viable counts from the same suspension on the various types of media tested showed that compared to nutrient agar, growth of both F89 and SN109 strains on the minimal medium (MM) with glycerol as the carbon source was decreased by approximately 2 logs, while on minimal medium with Fe^{+++} only by approximately 0.5 log. Growth appears on nutrient agar after 8 days but 19 days were required before individual colonies became visible to the unaided eye on MM even with Fe^{+++}. All tests were done in quadruplicate.

2. *Tests for viability following refrigeration.* Viable counts on nutrient agar of *M. phlei* strains after various periods at 4 C (Table 4) provided evidence that no appreciable loss of viability occurred even after 10 days of refrigeration. All tests were performed in quadruplicate.

3. *Bacterial survival following phage infection.* Viable counts made on nutrient agar of bacterium-phage mixtures at various multiplicities of infection following incubation at 37 C for 24 hr established that phage Bo2 at an MOI of 1 killed less than 90 percent of a susceptible *M. phlei* F89 population.

D. *Transduction of ability to utilize D-xylose as sole carbon source.*

Preliminary experiments were performed employing phage Bo2 propagated on strain SN109 (hereafter designated as Bo2·SN109) as the transducing phage and strains F89 wild type, as the recipient. Sterile phage preparations, not pretreated with DNase, were then used to infect the recipient using various multiplicities of infection. The phage-bacterium mixtures were incubated for 48 hr and subsequently inoculated onto the selective medium, MM containing

TABLE 4. Viability of *M. phlei* strains F89 and SN109 after incubation at 4 C.

Time (days)	Viable count (CFU/ml) Strain F89	Strain SN109
0	3.8×10^7	2.6×10^7
3	4.1×10^7	2.4×10^7
5	3.2×10^7	2.8×10^7
7	3.5×10^7	2.2×10^7
10	3.5×10^7	2.5×10^7

All tests were performed in quadruplicate

$FeCl_3$ but with no streptomycin. Controls employed in this experiment were bacteria incubated with sterile HIB in place of phage and bacteria incubated with phage Bo2 which had never been propagated on strain SN109 but instead had been propagated exclusively on strain F89. The results of these preliminary experiments (Table 5) established that while the tube containing bacteria incubated with phage Bo2·SN109 at an MOI of 1.0 produced 328 colonies per ml that were able to utilize xylose as a sole carbon source, 16 xyl^+ colonies per ml were obtained from the incubation mixture containing phage Bo2·SN109 at an MOI of 0.1, 5 xyl^+ colonies per ml from the one containing phage Bo2·F89 and 14 xyl^+ colonies per ml from the control employing sterile HIB in place of phage. Although all of these mutants were apparently able to utilize xylose as a sole carbon source, they did not produce sufficient acid to convert bromcresol purple to its yellow acidic form which the donor strain SN109 was capable of doing (Table 3a). Microcolonies (Fig. 1) which appeared after 4 weeks, but were not clearly visible to the naked eye, were not counted in any experiments since subsequent tests revealed that they could not utilize xylose as a sole carbon source.

The next set of experiments contained an additional control. A mutant of *M. phlei* F89 resistant to over 100 µg/ml streptomycin (xyl^-, str-r) was employed as the recipient. When compared by biochemical and other tests to wild strain F89, the mutant produced identical reactions with the single exception of resistance to >100 µg/ml streptomycin. The selective medium employed was MM with $FeCl_3$ and 50 µg/ml streptomycin sulfate. Neither the donor *M. phlei* SN109 (xyl^+, str-s) or recipient *M. phlei* F89 (xyl^-, str-r) could grow on this medium, with the exception of spontaneous mutants and

TABLE 5. Orientation experiments for genetic transfer.

Phage used as donor*	Initial MOI	Xyl^+CFU/ml†
Bo2 SN109	1.0	328
Bo2 SN109	0.1	16
Bo2 F89	1.0	5
Control: sterile HIB	0	14

*F89 (xyl^-, str-s) served as the recipient strain. Its titer was 6.6 x 10^8 CFU/ml.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

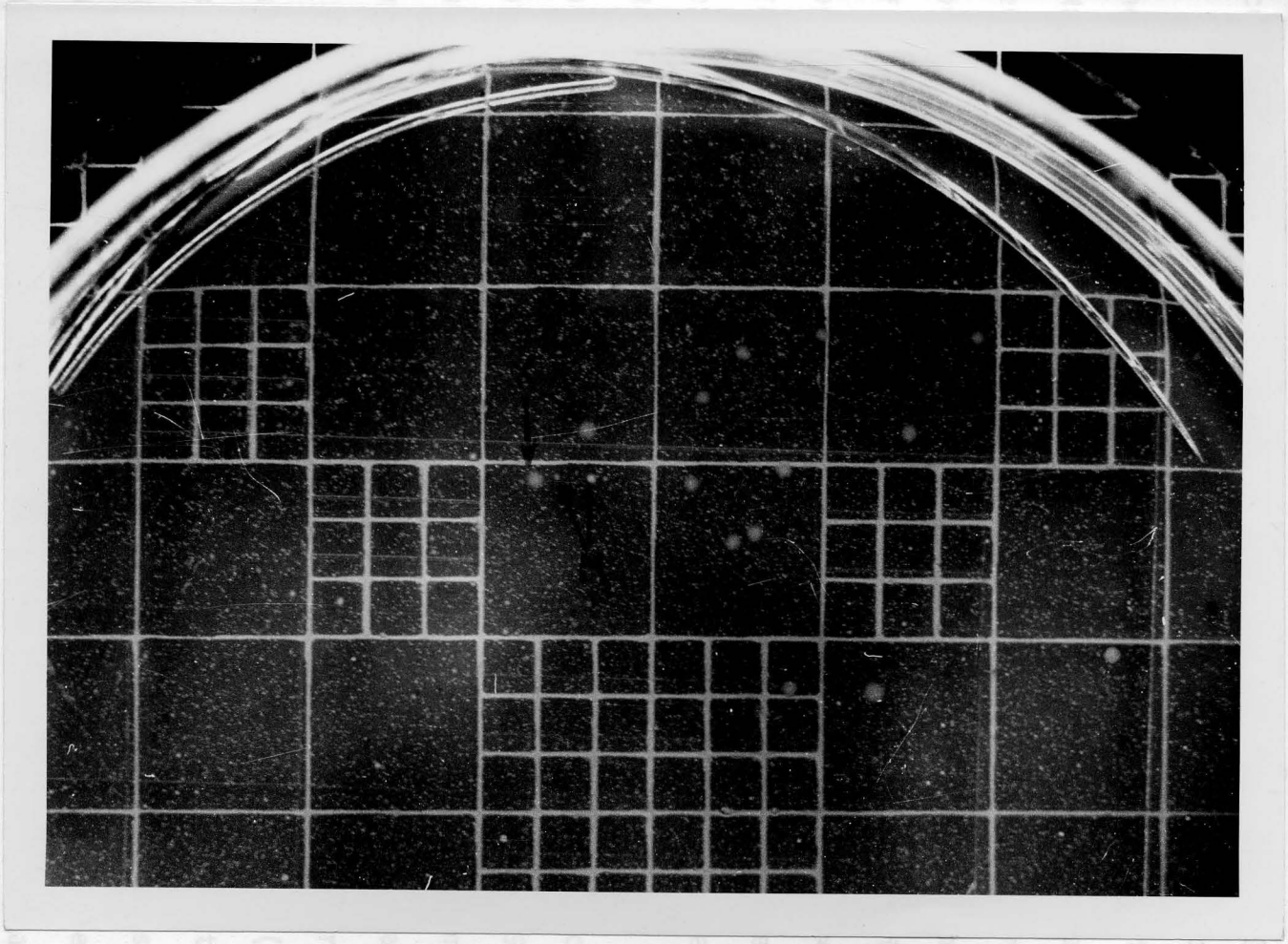

Fig. 1. Photograph of colonies growing on medium containing xylose as sole carbon source (x2). In contrast to the large colonies (X), microcolonies (M) were not able to utilize xylose when repropagated, washed and replated, therefore they can not be considered of the xyl^+ genotype.

phenocopies. Various MOIs were employed. The phage-bacterium mixtures were incubated for 48 hr and subsequently inoculated onto the selective medium. The results of these experiments (Table 6) established that genetic transfer occurred when the concentration of phage was 1×10^8 PFU or higher.

The next set of experiments contained controls designed to establish whether or not the mechanism of genetic transfer was transduction. First DNase was employed to remove DNA from the sterile phage preparations, and second the phage were heat inactivated by autoclaving to eliminate all plaque forming activity. The experiments were performed using the same conditions of incubating the recipient bacterial cultures with phage or with the appropriate control for 48 hr at 37 C. The results of these experiments (Table 7) show that 1) the incubation mixture containing untreated phage Bo2·SN109 at a titer of 4×10^8 PFU/ml yielded 226 xyl^+ colonies/ml; 2) only 184 such colonies/ml were obtained from the incubation mixture containing phage Bo2·SN109 pretreated with 25 µg/ml DNase; and 3) 54 colonies/ml were obtained from the incubation mixture containing phage Bo2·SN109 that had been autoclaved. To confirm these unexpected results, these experiments were repeated with the following modifications: heat killed phage rather than viable phage was treated with DNase, and as an additional control, sterile HIB in place of phage was used. The results of these experiments (Table 8) were in agreement with earlier results. The incubation mixture containing untreated phage Bo2·SN109 at a titer of 8×10^8 PFU/ml gave rise to 515 xyl^+ colonies/ml while the one containing autoclaved phage still yielded 172 colonies/ml. However, mixing the recipient population with phage which had first been autoclaved and then treated with DNase produced essentially the

TABLE 6. Genetic transfer with phage Bo2·SN109 using str-r mutant of strain F89 as recipient.

Incubation mixture*	Initial MOI	Xyl$^+$CFU/ml†
1	5.0	232
2	1.0	108
3	0.1	10
Control: sterile HIB	0.0	7

*The titer of the recipient strain was 1.0×10^8 CFU/ml.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

TABLE 7. Effect of DNase treatment and autoclaving on the transfer activity of phage Bo2·SN109.

Phage preparation*	Initial MOI	Xyl^+CFU/ml†
1. Untreated	1.0	226
2. Pretreated with DNase	1.0	184
3. Autoclaved	0	54

*F89 (xyl^-, str-r) served as the recipient strain. Its titer was 4.0×10^8 CFU/ml.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

TABLE 8. Effect of autoclaving and subsequent DNase treatment on transfer activity of phage Bo2·SN109.

Phage preparation*	Initial MOI	Xyl+CFU/ml†
Untreated	2.0	515
Autoclaved	0.0	172
Autoclaved and DNase treated	0.0	28
Control: sterile HIB	0.0	19

*F89 (xyl^-, str-r) served as the recipient strain. Its titer was 4.0×10^8 CFU/ml.

†Number of colonies obtained per 1.0 ml incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

same yield of xyl^+ colonies as the control. Boiling at 99.5 ± 0.5 C as a method of inactivating phage instead of autoclaving, was also tried. The results of these experiments (Table 9) confirmed that heat killed phage retained some transfer activity.

The 48 hr incubation procedure employed in all of these experiments had one major drawback. Both phage and bacteria could multiply, thereby making any calculations of spontaneous mutation frequency or frequency of genetic transfer invalid. To avoid this problem subsequent experiments were modified by reducing the incubation period of recipient bacterial culture and phage to an interval which would permit neither phage nor bacterial multiplication. Furthermore, the phage preparations were treated with DNase to eliminate any transfer by free DNA and the selective medium was modified to its final form MM'. The first experiments of this series employed a 24 hr culture of strain F89 which was incubated with phage Bo2·SN109 pretreated with DNase for 1 hr; no transduction occurred. This indicated that not all phases of bacterial growth permitted transduction.

Experiments were then designed to find those phases of bacterial growth which permitted transduction. The growth curve of *M. phlei* F89 (xyl^-, str-r) (Fig. 2) shows that at 24 hr the culture is in early log phase, since under the conditions performed, the lag period is 16-20 hr and stationary phase begins after about 48 hr. The generation time is approximately 5-6 hr for log phase cells. In the next transduction experiments different phases of bacterial growth were employed: a 2 hr culture representing lag phase, a 24 hr culture representing early log phase, and a 48 hr culture representing late log-early stationary phase. Bacteria were incubated with phages for 1 hr

TABLE 9. Effect of DNase and heating on transfer activity of phage Bo2·SN109.

Phage preparation*	Initial MOI	Xyl$^+$CFU/ml†
1. Untreated	1.0	240
2. Pretreated with DNase	1.0	206
3. Heated to 99.5 ± 0.5 C (but not treated with DNase)	0.0	42

*F89 (xyl$^-$, str-r) served as the recipient strain. Its titer was 3.0×10^8 CFU/ml.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

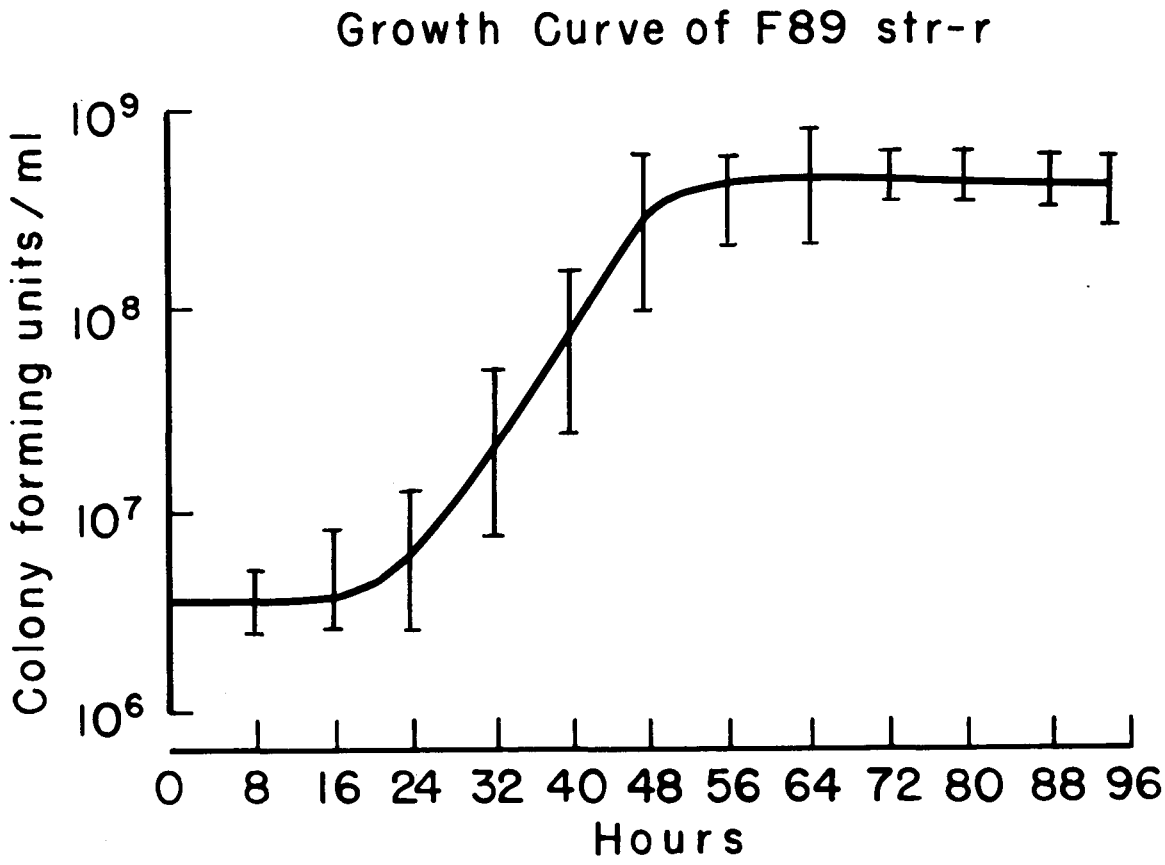

Fig. 2. Growth curve of the recipient strain *M. phlei* F89 str-r.

at 37 C. The results (Table 10) show that transduction occurred only when 48 hr cultures were used. All controls, those containing heat killed phage or no phage, produced essentially the same number of colonies. The next experiment was an elaboration upon the first experiment designed to show which phases of bacterial growth permitted transduction. Phage Bo2·SN109 was pretreated with DNase and incubated for 30 min with recipient cultures of various ages. The results (Table 11) proved that the bacteria had to be approximately 40 hr old for transduction to occur but that cells 48 hr or older seemed to work best. Again it was clearly shown that no transfer occurred when 24 hr cultures were employed. When 32 hr cultures were used only slight transfer activity could be demonstrated.

Another question to be answered was the effect of incubation time on transduction. To answer this our next experiment used phage preparations pretreated with DNase and incubated for various periods of time with a 48 hr culture of recipient bacteria. Controls employed were phages mixed with bacteria but immediately plated out and sterile HIB in place of phage incubated with bacteria. The results (Table 12) demonstrated that 15 min allowed for bacterium phage contact following thorough mixing was sufficient for optimal transduction. The two controls yielded approximately the same number of colonies/ml.

As a result of these last three sets of experiments all subsequent transduction experiments employed 48 hr recipient bacterial cultures which were incubated with phage for 30 min.

The next experiment was designed to establish that all genetic transfer in a system where the phage is pretreated with DNase was due to transduction.

TABLE 10. Transduction as a function of age of recipient culture. I. Orientation experiment.

Phage preparation*	Age of culture(hr)**	MOI	Xyl$^+$CFU/ml†
Bo2·SN109	2	2.0	14
Bo2·SN109 autoclaved	2	0.0	11
Bo2·SN109	24	2.0	9
Bo2·SN109 autoclaved	24	0.0	14
Bo2·SN109	48	2.0	108
Bo2·SN109 autoclaved	48	0.0	18
Sterile HIB in place of phage	48	0.0	15

*Phage preparation was pretreated with DNase.

**F89 (xyl$^-$, str-r) served as the recipient strain. Its titer was 3.0×10^8 CFU/ml.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 10 plates.

TABLE 11. Transduction as a function of age of recipient culture. II. Studies at 8 hr intervals.

Phage preparation*	Age of culture(hr)**	MOI	Xyl^+CFU/ml†
Bo2·SN109	24	1.1	25
Sterile HIB in place of phage	24	0.0	37
Bo2·SN109	32	0.9	27
Sterile HIB in place of phage	32	0.0	19
Bo2·SN109	41	1.5	66
Sterile HIB in place of phage	41	0.0	8
Bo2·SN109	48	1.8	174
Sterile HIB in place of phage	48	0.0	50
Bo2·SN109	56	1.8	133
Sterile HIB in place of phage	56	0.0	38

*Phage preparation was pretreated with DNase. Its titer was 6.0×10^8 PFU/ml.

**F89 (xyl^-, str-r) served as the recipient strain.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 10 plates.

TABLE 12. Effect of incubation time on transduction.

Incubation mixture*	Incubation time	Xyl$^+$CFU/ml†
1	0 min	22
2	15 min	107
3	30 min	116
4	60 min	99
5	120 min	101
Control**	120 min	19

*Titer of F89 (xyl$^-$, str-r) was 3.3 x 10^8 CFU/ml and the titer of phage Bo2·SN109 was 6.0 x 10^8 PFU/ml. Phage preparation was pretreated with DNase. Age of recipient culture was 48 hr.

**Sterile HIB in place of phage.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 10 plates.

Phage treated with antiphage serum was employed as an additional control. The results (Table 13) show that the incubation mixture containing viable phage Bo2·SN109 without antiphage serum (1) yielded 147 xyl^+ colonies/ml as compared to about 40 in the controls. The addition of antiphage Bo2 serum to phage Bo2·SN109 (2) or replacement of viable phage with either autoclaved phage (3) or HIB in place of phage (4) gave rise to essentially the same number of colonies.

The finding that recipient cultures had to be at a certain growth phase was surprising since it is not common to transduction, ahtough this is common to transformation. The possibility that some factor resulting from bacterial growth in the medium was necessary for transduction had to be investigated. A 24 hr culture of recipient bacteria was resuspended in the sterile filtrate of a 48 hr culture. This bacterial culture was then mixed with phage and incubated for 30 min at 37 C. Another sample was incubated for an additional 24 hr at 37 C. The results evinced that a) no transduction occurred and b) that the bacteria did not multiply to any extent in the 48 hr culture broth.

The frequency of transduction [xyl^+ CFU (corrected for spontaneous mutants)/input phage PFU] could be calculated from all genetic transfer experiments where multiplication of phage and bacteria in the incubation mixture was negligible. This was assumed for any experiment where the incubation time was 1 hr or less. Table 14 lists the calculated transduction frequencies based on data from Tables 9-12. The values obtained were between 1.4×10^{-7} and 2.0×10^{-7}. The average value for spontaneous mutation rates for the same experiments, calculated from the control in which HIB replaced phage, was 6.0×10^{-8}.

TABLE 13. Establishment of the transducing role of phage Bo2·SN109.

Phage preparation*	MOI	Xyl$^+$CFU/ml†
1. Bo2·SN109	0.9	147
2. Bo2·SN109 plus antiphage Bo2 serum	<0.1	40
3. Bo2·SN109 autoclaved	0.0	49
4. Sterile HIB in place of phage	0.0	41

*Phage preparation was pretreated with DNase. 48 hr recipient cultures were employed. Phage was incubated with bacteria for 30 min. Phage input was 6×10^8 PFU.

†Number of colonies obtained per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 10 plates.

TABLE 14. Transduction frequency*

Based on data from table no.	Phage input PFU**	Xyl^+CFU/ml†	Transduction Frequency
10	6×10^8	93	1.5×10^{-7}
11	6×10^8	124	2.0×10^{-7}
12(2)	6×10^8	88	1.5×10^{-7}
12(3)	6×10^8	97	1.6×10^{-7}
12(4)	6×10^8	80	1.4×10^{-7}
13	6×10^8	106	1.8×10^{-7}

*$\frac{xyl^+\text{CFU (corrected for spontaneous mutation)}}{\text{phage input}}$

**Phage were pretreated with DNase in all experiments. The MOI was approximately 1.

†Number of xyl^+ colonies obtained per 1.0 ml corrected for spontaneous mutation. The age of recipient cultures was 48 hr in all experiments.

E. *Characterization of xyl$^+$ colonies.* In order to exclude carry-over of trace nutrients or phenocopy, approximately 540 of all xyl$^+$ colonies were randomly selected from an experiment in which a) untreated phage, b) heat-killed phage and c) heat-killed phage treated with DNase and HIB in place of phage were employed. Two types of colonies, the one clearly visible to the naked eye (1-2 mm in diameter) and the other hardly visible to the naked eye (less than 0.5 mm) were prepared for detailed analysis by propagation on nutrient agar and subsequent thorough washing. All strains which passed the preliminary tests of acid fastness, growth at 52 C, and ability to utilize xylose as sole carbon source were further examined by the amidase and carbohydrate-nitrite tests and also tested for lysogeny by two criteria, production of phage and superinfection immunity to phage Bo2. All of the colonies tested were acid fast and could grow at 52 C, however only 80% in category a), 60% in category b) and 20% in category c) and none of the microcolonies could utilize xylose as a sole carbon source. A comparison of the colonies tested to xyl$^+$ donor SN109 organisms (Table 15) shows that although all the resulting strains could utilize xylose as a sole carbon source, none could produce acid from xylose or use xylose as an electron donor for the reduction of nitrite except the donor strain SN109. When tested for lysogeny strains from group a with one exception produced plaques when tested on a susceptible strain F89, and all of these were immune to superinfection by phage Bo2, while in contrast none of the strains from groups b and c were lysogenic.

In addition, the results of the amidase and carbohydrate-nitrite tests were identical for all strains, with the single exception of xylose in the latter.

TABLE 15a. Characterization of representative colonies obtained in transduction experiments after washing and replating.

Group*	Strain	Test						
		Acid fastness	Growth at 52 C	Use of xylose as carbon source	Production of acid from xylose	Use of xylose as electron donor for nitrite reduction	Production of phage	Susceptibility to phage Bo2·SN109
Donor	SN109	+	+	+	-	+	-	+
Recipient	F89 (str-r)	+	+	-	-	-	+	+
a	F89 (xyl^+) I	+	+	+	-	-	+	-
a	F89 (xyl^+) II	+	+	+	-	-	+	-
a	F89 (xyl^+) III	+	+	+	-	-	+	-
a	F89 (xyl^+) IV	+	+	+	-	-	+	-
a	F89 (xyl^+) V	+	+	+	-	-	+	-
a	F89 (xyl^+) VI	+	+	+	-	-	+	-
a	F89 (xyl^+) VII	+	+	+	-	-	+	-
a	F89 (xyl^+) VIII	+	+	+	-	-	+	-
b	F89 (xyl^+) 1	+	+	+	-	-	-	-
b	F89 (xyl^+) 2	+	+	+	-	-	-	-
b	F89 (xyl^+) 3	+	+	+	-	-	-	-
c	F89 (xyl^+) A	+	+	+	-	-	-	-

*
a. from incubation mixture containing viable phage
b. from incubation mixture containing heat killed phage
c. from incubation mixture containing sterile HIB in place of phage

F. *High frequency transduction of the ability to utilize xylose as a sole carbon source.* Lysogenic (xyl^+) transductants (Table 15) were subsequently employed as donors for high frequency transduction experiments. Of the seven strains that produced phage, four produced titers of about 10^4 PFU/ml. DNase-treated sterile filtrates of these strains were incubated with recipient F89 (xyl^-, str-r) for 30 min at 37 C. The results of these experiments (Table 16) showed that the incubation mixtures containing phage from xyl^+ transductant colonies II, V and VIII yielded 66, 32, and 80 colonies/ml respectively, while the incubation mixtures containing phage from transductant III and sterile HIB in place of phage yielded no colonies on selective medium MM'.

Followup experiments employed fresh batches of phage from colonies II, V, and VIII utilizing various multiplicities of infection ranging from 1 to 10^{-4}. The results of these experiments (Table 17) confirmed that all three lysates could transduce the ability of utilizing xylose as a sole carbon source at very high frequencies.

Additional high frequency transduction experiments were designed with the purpose of eliminating the possibility of free DNA interfering with the results of transduction. HFT lysate from transductant colony VIII was employed as the transducing lysate. The phage was pretreated with DNase prior to incubation with bacteria. DNase was added again at the time of mixing phage with bacteria at a final concentration of 25 µg/ml. After the incubation mixture was filtered and the bacteria collected on the filter and washed, the bacteria were resuspended in a saline solution containing 25 µg/ml DNase prior to plating. Two controls were employed; one used no phage, while the other used no DNase subsequent to initial treatment of phage. The results of

TABLE 15b. Characterization of representative colonies obtained in transduction experiments after washing and replating.

		Amidase Test											Carbohydrate-nitrite Test																	
Group*	Strain	Acetamidase	Benzamidase	Urease	Isonicotinamidase	Nicotinamidase	Pyrazinamidase	Salicylamidase	Allantoinase	Succinamidase	Malonamidase	Control (0.85% NaCl)	Glucose	Rhamnose	Xylose	Arabinose	Mannose	Galactose	Mannitol	Sorbitol	Inositol	Dulcitol	Lactose	Raffinose	Maltose	Trehalose	Fructose	Sucrose	Glycerol	Control (no carbohydrate)
Donor	SN109	+	-	+	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
Recipient	F89 (str-n)	+	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) I	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) II	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) III	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) IV	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) V	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) VI	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) VII	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
a	F89 (xyl^+) VIII	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
b	F89 (xyl^+) 1	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
b	F89 (xyl^+) 2	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
b	F89 (xyl^+) 3	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
c	F89 (xyl^+) A	(+)	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-

*a. from incubation mixture containing viable phage
b. from incubation mixture containing heat killed phage
c. from incubation mixture containing HIB in place of phage

TABLE 16. High frequency transduction obtained with phage isolated from lysogenic xyl^+ transductants. I. Orientation experiment.

Source of strain*	Phage input PFU/ml	MOI†	Xyl^+ CFU/ml††
F89 (xyl^+) II	1.1×10^4	1.1	66
F89 (xyl^+) III	1.2×10^4	1.2	0
F89 (xyl^+) V	1.0×10^4	1.0	32
F89 (xyl^+) VIII	1.5×10^4	1.5	80
Control**	0	-	0

*See Table 15

**Control consists of bacteria and sterile HIB.

†48 hr recipient cultures were used in all experiments.

††Number of colonies per 1.0 ml of incubation mixture found on plates containing xylose as a sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

TABLE 17. High frequency transduction obtained with phage isolated from lysogenic xyl^+ transductants. II. Utilizing various multiplicities of infection.

Source transductant	Phage input (PFU/ml)	MOI	Xyl^+ CFU/ml*
II	1.5×10^4	1	20
	1.5×10^4	10^{-2}	28
	1.5×10^4	10^{-4}	132
V	1.3×10^4	1	20
	1.3×10^4	10^{-2}	60
	1.3×10^4	10^{-4}	106
VIII	1.2×10^4	1	34
	1.2×10^4	10^{-2}	94
	1.2×10^4	10^{-4}	172
Control**	0	0	6

*Number of colonies per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

**HIB replaced phage. CFU/ml of recipient culture was 1.5×10^8.

this experiment (Table 18) evince that there was essentially no difference between the results of the experiment utilizing DNase throughout all stages (42 xyl^+ colonies/ml) and the one in which DNase only was used in the pretreatment of phage (40 xyl^+ colonies/ml). In the control containing no phage but recipient bacterium and DNase no xyl^+ colonies were obtained.

One characteristic feature of HFT lysates which differed from that of the LFT lysate was their inability to retain transfer activity when stored at 4 C for a period longer than two weeks. In successive experiments with the same batch of HFT, consistently lower yields were obtained although the plaque forming activity remained fairly constant.

G. *Characterization of xyl^+ colonies obtained from high frequency transduction experiments*. Random selection was made of 12 xyl^+ colonies from the experiments of Table 15. These were grown on Löwenstein-Jensen medium at 52 C, harvested, washed in physiological saline and replated onto xylose minimal medium. Of 12 colonies selected, all were acid fast and could grow on Löwenstein-Jensen medium at 52 C, but only 11 could utilize xylose as a sole carbon source when replated onto selective medium. These 11 were further tested by basic tests (Table 19) as well as by the amidase tests (Table 20) and the carbohydrate-nitrite tests (Table 21). They were shown to possess characteristics identical to the parent strain except for their ability to utilize xylose as a sole carbon source and immunity to superinfection by phage Bo2. However, this second set of transductants did not produce phage.

In order to assess if the original transductant colonies from which the HFT lysates were derived were true or pseudolysogens, the following experiments were carried out: 0.1 aliquots of suspensions of the four

TABLE 18. Effect of DNase on High Frequency Transduction.

Donor HFT lysate	Phage titer (PFU/ml)*	DNase**	Xyl^+ CFU/ml†
VIII	1.2×10^4	-	40
VIII	1.2×10^4	+	42
Sterile HIB	0	+	0

*Recipient titer was 8.7×10^7 CFU/ml in all experiments.

**All phage preparations were pretreated with DNase (+) indicates addition of DNase to incubation mixture and suspension of washed cells in DNase, prior to plating onto the selective medium.

†Number of colonies per 1.0 ml of incubation mixture on plates containing xylose as the sole carbon source. Numbers in column represent the average of data obtained from 20 plates.

TABLE 19. Basic characterization of HFT transductants and the parent strain *M. phlei* F89 str-r.

Strain	Test						
	Acid fast	Growth at 52 C	Utilization of xylose as sole carbon source	Production of acid from xylose	Use of xylose as electron donor for nitrite reduction	Production of phage	Susceptibility to phage Bo2
M. phlei F89 str-r	+	+	-	-	-	-	+
HFT #1	+	+	+	-	-	-	-
HFT #2	+	+	+	-	-	-	-
HFT #3	+	+	+	-	-	-	-
HFT #4	+	+	+	-	-	-	-
HFT #5	+	+	+	-	-	-	-
HFT #6	+	+	+	-	-	-	-
HFT #7	+	+	+	-	-	-	-
HFT #8	+	+	+	-	-	-	-
HFT #9	+	+	+	-	-	-	-
HFT #10	+	+	+	-	-	-	-
HFT #11	+	+	+	-	-	-	-

TABLE 20. Comparison of HFT transductants and strain F89 str-r by the amidase test.

Strain	Amidase										
	Acetamidase	Benzamidase	Urease	Isonicotinamidase	Nicotinamidase	Pyrazinamidase	Salicylamidase	Allantoinase	Succinamidase	Malonamidase	Control (0.85% NaCl)
M. phlei F89 str-r	(+)	-	+	-	+	+	-	-	-	-	-
HFT #1	(+)	-	+	-	+	+	-	-	-	-	-
HFT #2	(+)	-	+	-	+	+	-	-	-	-	-
HFT #3	(+)	-	+	-	+	+	-	-	-	-	-
HFT #4	(+)	-	+	-	+	+	-	-	-	-	-
HFT #5	(+)	-	+	-	+	+	-	-	-	-	-
HFT #6	(+)	-	+	-	+	+	-	-	-	-	-
HFT #7	(+)	-	+	-	+	+	-	-	-	-	-
HFT #8	(+)	-	+	-	+	+	-	-	-	-	-
HFT #9	(+)	-	+	-	+	+	-	-	-	-	-
HFT #10	(+)	-	+	-	+	+	-	-	-	-	-
HFT #11	(+)	-	+	-	+	+	-	-	-	-	-

TABLE 21. Comparison of HFT transductants and the strain F89 str-r by the carbohydrate-nitrite test.

Strain	Carbohydrate																				
	Glucose	Rhamnose	Xylose	Arabinose	Mannose	Galactose	Mannitol	Sorbitol	Inositol	Dulcitol	Lactose	Raffinose	Maltose	Trehalose	Fructose	Sucrose	Glycerol	Control (no carbohydrate)
M. phlei F89 str-r	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #1	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #2	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #3	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #4	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #5	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #6	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #7	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #8	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #9	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #10	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-
HFT #11	+	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	-

colonies (Table 16) which had produced a titer of phage as high as 10^4 PFU/ml were inoculated into 0.9 ml of HIB containing 0.05% Tween-80 and into 0.9 ml antiphage serum. The former was incubated for 48 hr at 37 C and then reinoculated into fresh HIB containing 0.05% Tween-80 and the entire process was repeated three times. The latter was incubated for 96 hr after which both were plated onto susceptible F89 indicator strain to test for plaque production. Tween-80 did not prevent plaque production. However, incubation with antiphage serum did eliminate plaque production in one of the four strains, HFT colony III. This strain was the one that indicated no high frequency transduction capability although it produced yields of phage as high as 1.2×10^4 PFU/ml. A similar experiment, in which both bacteria and phage were quantitated was then performed. Each of the four strains was incubated at 37 C for 96 hr in antiphage Bo2 serum and in normal serum. Bacteria were collected on millipore filters and washed then resuspended in HIB and reincubated for an additional 48 hr. Viable counts of the bacteria and plaque counts of phage were then made. Results of these experiments (Table 22) confirmed the findings of the previous experiment. As expected, antiphage serum decreased phage output by preventing reinfection and subsequent multiplication of liberated phages. However, three of the four strains could not be cured which indicated that they were lysogenic, in contrast to the fourth strain. Upon repropagation, the pseudolysogenic fourth strain did not regain the ability to produce phage, which further indicated that it was cured.

H. *Transfer of additional markers in M. phlei and use of other phages for transduction in other systems.* Attempts to transfer drug resistance from one

TABLE 22. Effect of antiphage Bo2 serum on phage production by transductants.

Source strain*	Phage count (PFU/ml)†	
	Antiphage serum	Normal serum
F89 xyl^+ II	20	240
F89 xyl^+ III	0	680
F89 xyl^+ V	80	450
F89 xyl^+ VIII	45	170

* Initial inoculum was 1.0×10^6 CFU/ml for each strain. After 96 hr in antiserum or normal serum, cells were collected on millipore filters washed and reincubated at 37 C in sterile HIB for 48 hr.

† Counts represent phage titer of resuspended cells after 48 hr of incubation in fresh medium.

M. phlei strain to another utilizing phage Bo2 failed. Attempts to develop auxotrophs of strain SN109 by employing nitrosoguanidine as a mutagen have thus far not produced mutants of sufficiently low back mutation rate to be employed in transduction experiments with phage Bo2·SN109. Back mutation rate for SN109 (str-r) → SN109 (str-s) was 4×10^{-8} and for cycloserine resistant SN109 → cycloserine sensitive SN109 was 1×10^{-8}. Phage Bol was found to be unsuitable for transduction due to its high rate of lysis. In contrast to phlei phage Bo2 which killed less than 90% of susceptible *M. phlei* at an MOI as high as 1.0, smegmatis phage Bol killed greater than 90% of a susceptible population of *M. smegmatis* at an MOI as low as 0.006. Propagation of Bol lysogenically on the donor did not noticeably alter its lytic activity.

I. *Characterization of phage Bo2·SN109.* Phage Bo2 that had been propagated on strain SN109 was characterized for plaque morphology, host range, DNA base composition, single step growth curve characteristics, adsorption rates, single burst size and electron microscopic morphology.

1. *Plaque morphology after three days.* Phage stocks were diluted so that there were 30-50 single plaques per plate that were well separated and easily observable. After three days phage Bo2·SN109 formed circular plaques 3-4 mm in diameter (Fig. 3). These plaques were quite hazy, due to the presence of many survivor bacteria within the plaque. This was demonstrated by scraping across the center of the plaque with a sterile bacteriological needle and inoculating a Löwenstein-Jensen plate with the scrapings. In each instance there was growth.

2. *Host range determination.* Thirty-three different strains, including ten different species of *Mycobacterium*, were tested for their susceptibility

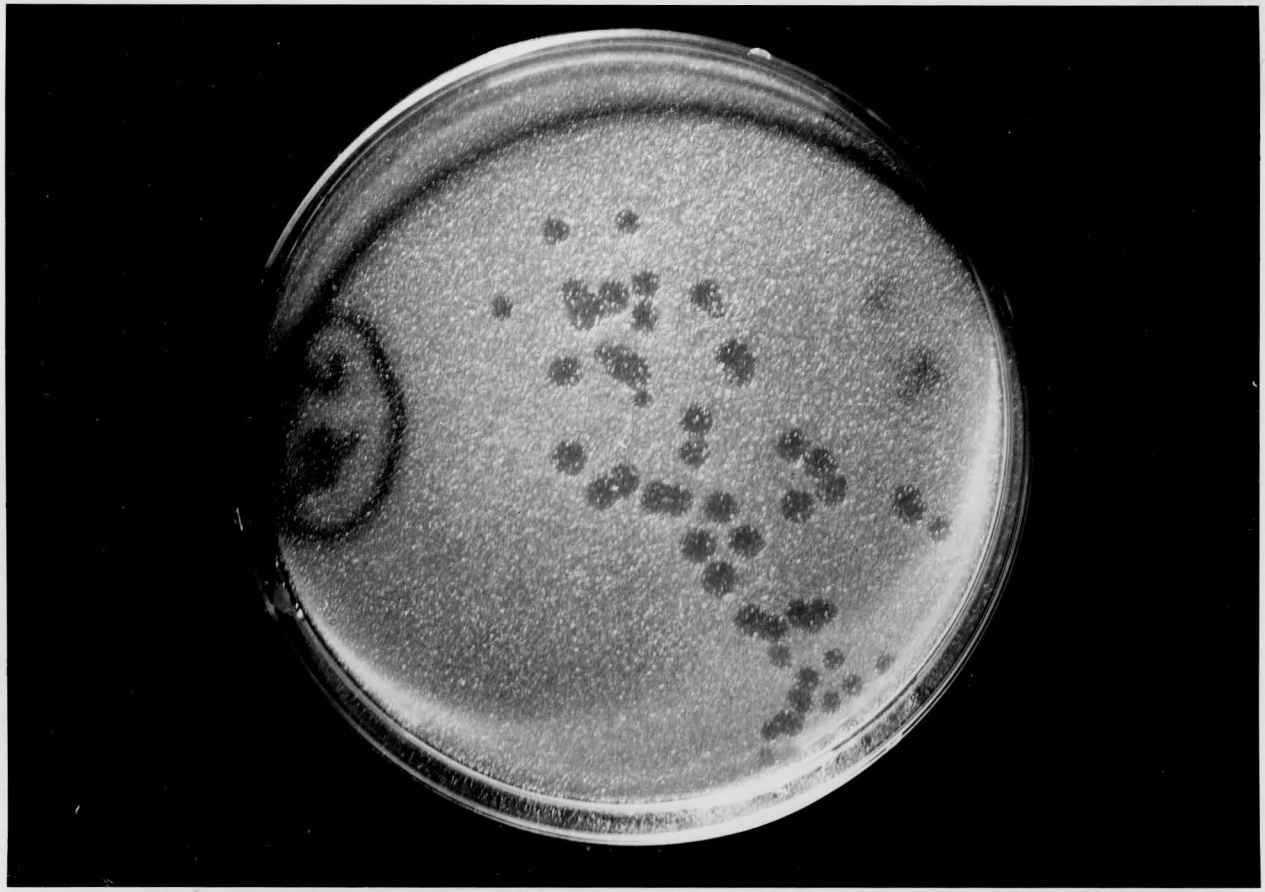

Fig. 3. Plaque morphology of phage Bo2 SN109 after three days of incubation at 37 C. Note that bacterial growth can be seen within each plaque.

to lysis by 10^7 PFU/ml of phage Bo2·SN109. It can be seen from the results (Table 23) that phage Bo2 lysed all strains of *M. phlei*, four of the five *M. smegmatis* strains, and one of the two *M. avium* strains. The relative efficiency of plating is shown in Table 24. The efficiency of plating was greatest when the phage was plated on its host strain. A study was also made of the host range of phage Bo2·SN109 compared to the host range of phage Bo2·F89, the original phage obtained from Borstel. The results (Table 25) showed that whereas phage Bo2 passed once on SN109 could lyse several strains of *M. smegmatis* as well as all strains of *M. phlei*, Bo2·F89 could lyse only *M. phlei* strains.

3. *DNA base composition.* The GC content of phage Bo2·SN109 was 72.0%, as determined by buoyant density in CsCl, identical to its host strain SN109 and also to *Micrococcus lysodeikticus* DNA standard used as a marker. All bands were unaffected by RNase treatment but disappeared following treatment with DNase.

4. *Single step growth curves.* The results of the single step growth experiments on phage Bo2 propagated on SN109 (Fig. 4) showed that phage Bo2·SN109 had a latent period of 260 min, a rise period of 180 min and a burst size of about 10. The addition of Tween-80 delayed the rise and latent periods, but did not significantly change the burst size.

5. *Adsorption studies.* Adsorption experiments were attempted at an MOI of 0.01 using both 24 hr and 48 hr cultures of strain SN109. After 1 hr the degree of adsorption was 38% when a 24 hr culture of strain SN109 was employed and 35% when a 48 hr culture of strain SN109 was employed. Using an MOI of 0.001 with a 24 hr culture of SN109 the degree of adsorption was 56%

TABLE 23. Host range of phage Bo2·SN109.

Strains*	Susceptibility to lysis by phage (10^7PFU/ml)
M. phlei SN101	+
M. phlei SN102	+
M. phlei SN103	+
M. phlei SN104	+
M. phlei SN105	+
M. phlei SN106	+
M. phlei SN107	+
M. phlei SN108	+
M. phlei SN109	+
M. phlei SN110	+
M. phlei SN111	+
M. phlei SN112	+
M. phlei SN113	+
M. phlei SN114	+
M. phlei SN115	+
M. phlei SN118	+
M. phlei SN119	+
M. phlei F89	+
M. vaccae SN920	-
M. smegmatis SN2	+
M. smegmatis SN5	+
M. smegmatis SN13	-
M. smegmatis SN38	+
M. smegmatis SN46	+
M. fortuitum SN203	-
M. avium SN304	+
M. avium SN327	-
Battey group III SN403	-
Group II Scotochromogen SN650	-
Group II scotochromogen SN703	-
Group I Photochromogen SN531	-
M. bovis BCG	-
M. tuberculosis H37Ra	-

*All strains were obtained from the Borstel Culture Collection, Borstel, West Germany.

TABLE 24. Relative efficiency of plating (REOP) of phage Bo2·SN109*.

Strain	PFU/ml	REOP
M. phlei SN109	5.3×10^8	1.00
M. phlei F89	4.3×10^8	0.81
M. smegmatis SN2	2.6×10^8	0.49
M. smegmatis SN5	2.3×10^8	0.43
M. smegmatis SN38	8.4×10^7	0.16
M. smegmatis SN46	4.3×10^7	0.08
M. avium SN304	9.1×10^7	0.17

*Number of plaques obtained on *M. phlei* SN109 is considered as 100% efficiency of plating. Results were based on duplicate tests.

TABLE 25. Host range of Bo2·SN109 as compared to that of Bo2·F89.

Strain	Activity of phage* Bo2·SN109	Bo2·F89
M. phlei F89	+	+
M. phlei SN109	+	+
M. smegmatis SN2	+	-
M. smegmatis SN5	+	-
M. smegmatis SN10	+	-
M. smegmatis SN13	-	-
M. smegmatis SN38	+	-
M. smegmatis SN46	+	-
M. smegmatis (Holland strain)	-	-
M. fortuitum SN203	-	-

*Phage concentration was 10^7 PFU/ml in all assays.

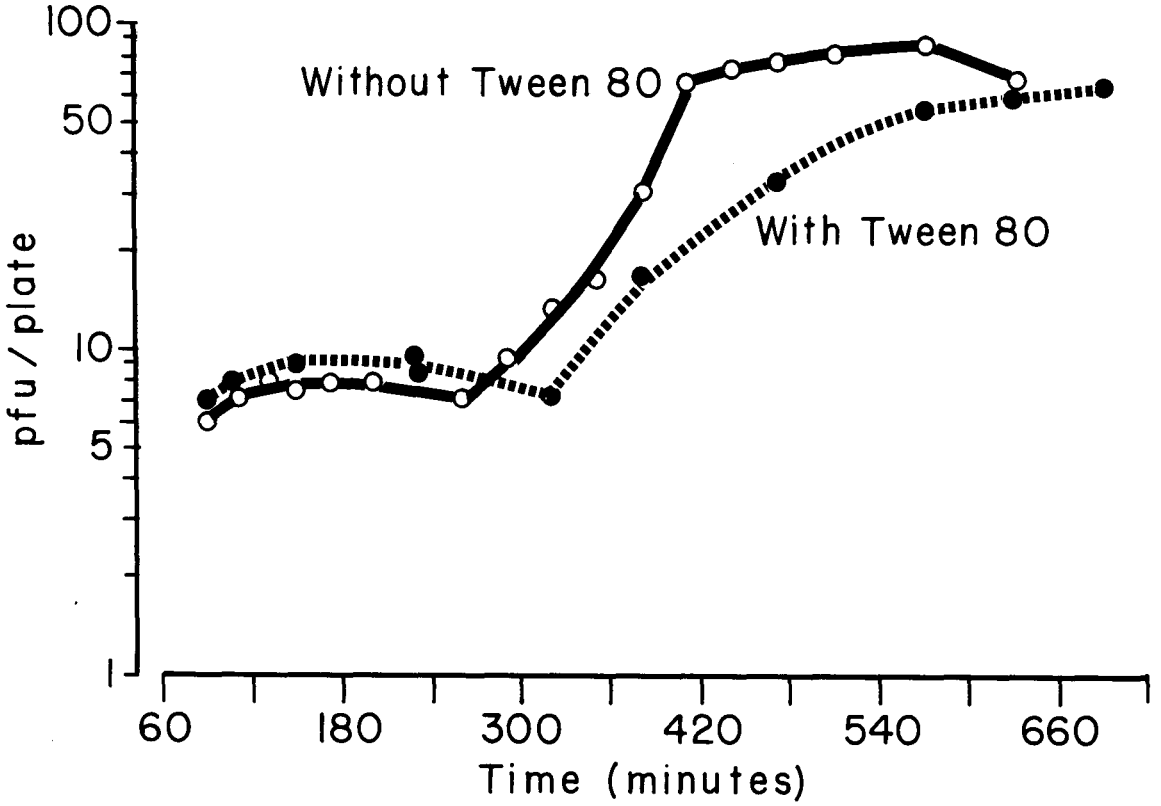

Fig. 4. Single step growth studies of phage Bo2·SN109 in HIB with, and without 0.05% Tween-80.

after 1 hr. Adsorption studies were also made employing strain F89 str-r at MOIs of 0.01 and 0.001 using both 24 and 48 hr cultures. At an MOI of 0.01 after 1 hr the degree of adsorption was 44% when a 24 hr culture was used and 58% when a 48 hr culture was used. At an MOI of 0.001 after 1 hr the degree of adsorption was 54% when a 24 hr culture was employed and 62% when a 48 hr culture was employed. These figures represent the average of four experiments, each in duplicate.

6. *Single burst studies.* The results of single burst experiments (Table 26) confirmed the low average burst of phage Bo2·SN109 and showed that not all bacteria produce a burst of greater than 1 even after 8 hr incubation.

Employing the Poisson equation $P_N = \frac{n^N e^{-n}}{N}$, where P_N is the probability of the burst arising from N colony forming units and e^{-n} equals the number of samples where no burst could be observed, i.e., where no infective centers were present, it can be shown that the data from Table 26 fits a Poisson distribution.

In experiment 1, 27 tubes gave plaques.

$$P_{(o)} = \frac{23}{50} = .46 = e^{-n} = e^{-0.78}$$

This indicates that there are about 0.8 infective centers per tube.

$$P_{(1)} = \frac{n \times e^{-n}}{1!} = \frac{.78 \times .46}{1} = 0.3588$$

This indicates that the plaques on 18 plates out of 27 were according to probability due to the burst resulting from 1 infective center.

$$P_{(2)} = \frac{n^2 \times e^{-n}}{2!} = \frac{(.78)^2 \times .46}{2} = 0.140$$

This shows that the plaques on 7 plates out of 27 were according to

TABLE 26. Single burst experiments.

Experiment 1 - Plaques per plate from each tube.

1. 0	11. 1	21. 0	31. 0	41. 1
2. 1	12. 0	22. 1	32. 0	42. 0
3. 0	13. 18	23. 11	33. 14	43. 0
4. 0	14. 3	24. 0	34. 23	44. 17
5. 1	15. 0	25. 17	35. 0	45. 1
6. 8	16. 0	26. 0	36. 17	46. 0
7. 0	17. 7	27. 1	37. 0	47. 18
8. 1	18. 0	28. 13	38. 4	48. 1
9. 15	19. 26	29. 0	39. 0	49. 0
10. 0	20. 0	30. 17	40. 9	50. 1

Experiment 2 - Plaques per plate from each tube.

1. 1	11. 0	21. 0	31. 0	41. 0
2. 0	12. 0	22. 0	32. 14	42. 0
3. 0	13. 0	23. 0	33. 0	43. 0
4. 11	14. 0	24. 13	34. 0	44. 0
5. 0	15. 5	25. 0	35. 0	45. 12
6. 8	16. 0	26. 1	36. 0	46. 0
7. 0	17. 0	27. 0	37. 4	47. 0
8. 0	18. 0	28. 0	38. 0	48. 1
9. 9	19. 0	29. 0	39. 6	49. 0
10. 0	20. 8	30. 14	40. 0	50. 0

probability due to the burst resulting from 2 infective centers.

$$P_{(3)} = \frac{n^3 \times e^{-n}}{3!} = \frac{(.78)^3 \times .46}{6} = 0.036$$

This demonstrates that the plaques on 1.5 plates (1 or 2) were according to probability due to the burst resulting from 3 infective centers.

$$P_{(4)} = \frac{n^4 \times e^{-n}}{4!} = \frac{(.78)^4 \times .46}{24} = 0.011$$

This implies that the plaques on at most 1 plate were according to probability due to the burst resulting from 4 infective centers.

In experiment 2, 14 tubes gave plaques.

$$P_{(o)} = \frac{36}{50} = .71 = e^{-n} = e^{-0.34}$$

This established that there are less than 0.5 infective centers per tube.

$$P_{(1)} = \frac{n \times e^{-n}}{1!} = \frac{.34 \times .71}{1} = 0.241$$

This means that the plaques on 12 of the 14 plates that had plaques were, according to probability, due to the burst resulting from 1 infective center. The ideal single burst experiment should have almost all results due to single bursts, i.e., plaques on plates resulting from the burst of 1 infective center.

$$P_{(2)} = \frac{n^2 \times e^{-n}}{2!} = 0.041$$

This indicates that according to probability 2 plates show a burst resulting from 2 infective centers.

$$P_{(3)} = \frac{n^3 \times e^{-n}}{3!} = 0.005$$

This demonstrates that less than 1 plate was according to probability due to a burst resulting from 3 or more infective centers.

7. *Electron microscopic morphology of phage Bo2·SN109.* The electron micrographs obtained (Fig. 5) depicted phage Bo2 as having a hexagonal head 54 nm in diameter and a tail 9 nm in width and 221 nm in length. The tail appeared to terminate in a knob-like structure.

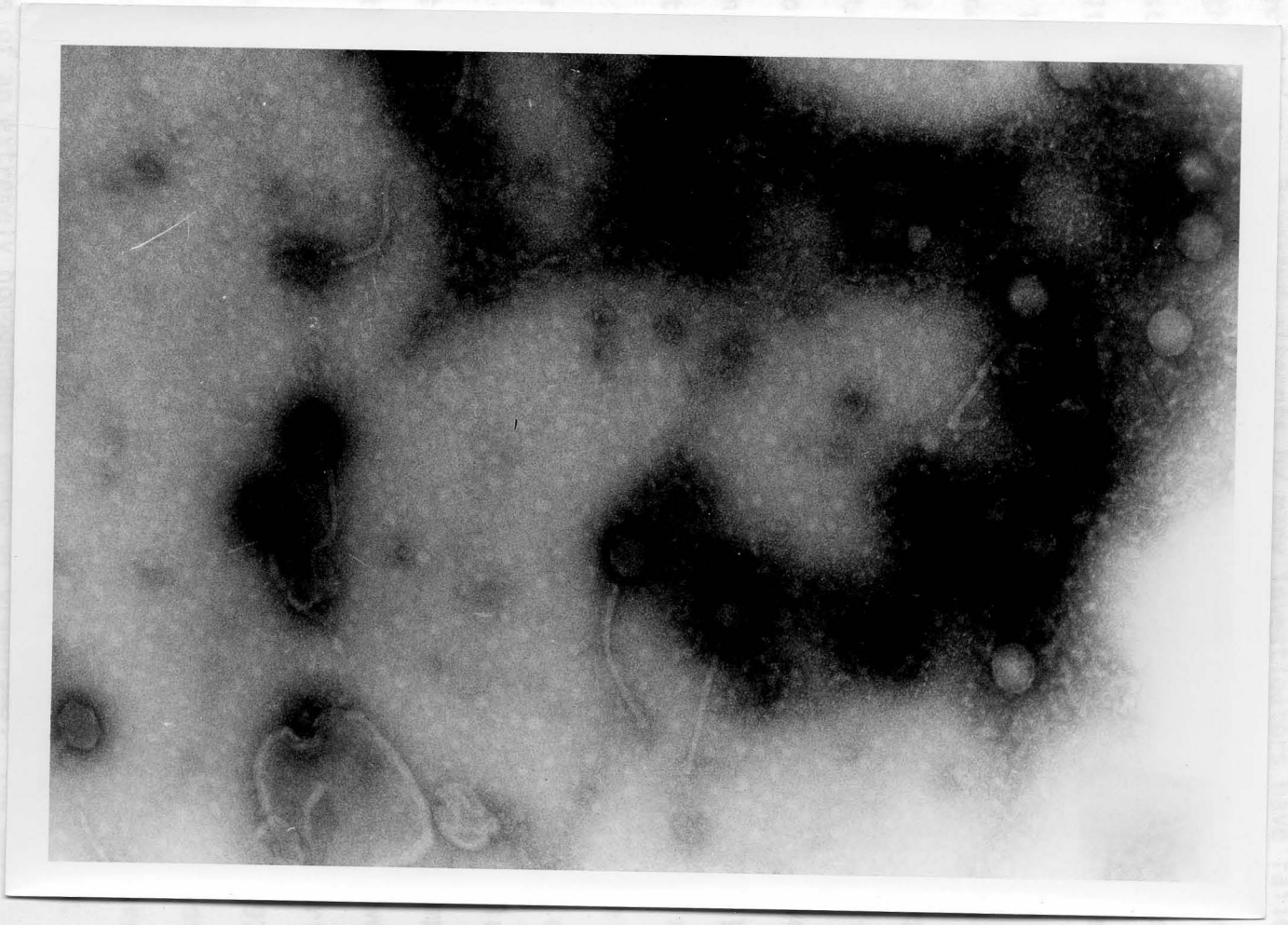

Fig. 5. Electron micrographs of phage Bo2·SN109 (x110,000).

DISCUSSION

A. *Introduction.* Repeated attempts by many investigators have failed to reveal the nature of a genetic transfer system(s) in the genus *Mycobacterium.* The reason for this is due mainly to technical problems. The first major difficulty in working with mycobacteria is quantitating the experiments. This is primarily due to rapid clumping of individual cells which is probably determined by their unique cell wall structure. The use of thorough mixing on a vortex type mixer throughout all phases of this study was an attempt to alleviate this technical problem. Another of the major problems is the slow growth of these organisms. Special precautions were necessary to avoid contamination and drying out of medium during prolonged cultivation periods. In addition methods to obtain reliable mutants of mycobacteria with adequate markers were not available until just recently (Holland and Ratledge, 1971).

B. *Characterization of Mycobacterium phlei strains.* Establishment of a genetic marker for *Mycobacterium phlei* was the first step to the solution of the problem of studying genetic transfer in mycobacteria. It was important to establish the identity of the strains employed in this study as well as that of any mutants before they could be used for this study. Unfortunately there have been several reports (Rytir et al., 1968; and Csillag, 1970) of strains of probable mycobacteria which were supposed to be mutants of *M. phlei* but which in fact could not be identified as such by standard taxonomic criteria. Many researchers have shown that the species *M. phlei* consists of an extremely homogeneous group of organisms. The most distinguishing character of this group is their ability to grow at 52 C within

7 days. The only other acid fast organism that possesses this property is *M. thermoresistibile* (Tsukamura, 1970) which can easily be differentiated from *M. phlei* by its carbohydrate fermentation pattern. However, to avoid any possibility of error a more thorough characterization of both the donor and recipient strains was made. The results of these tests for over 50 characters (Table 3) when compared to three major studies of the taxonomy of *M. phlei* (Bönicke, 1960: Tsukamura and Mizuno, 1968; Gordon and Mihm, 1959) shows with certainty that these strains are both *M. phlei*. The use of the amidase and carbohydrate-nitrite tests to identify transductants and other mutants once they had been established as acid fast bacteria and had shown that they could grow at 52 C conforms to the latest suggestion for identification of rapidly growing mycobacteria by rapid biochemical tests (Bönicke and Kazda, 1970). They have demonstrated that these two enzymatic tests provide a substantial spectrum of characters, suitable to identify any rapidly growing mycobacterial species known. If during the course of this study any "mutant" was found that did not conform to the above mentioned tests, it was discarded and not counted in the final results.

The results of the amidase test and carbohydrate-nitrite test obtained in this study agreed fully with those published by Bönicke (1960) and Bönicke and Kazda (1970) for the donor *M. phlei* SN109 strain. These authors did not examine *M. phlei* F89. Separation of the characters of utilizing xylose as a sole carbon source and production of acid from xylose was shown in the present study. Transductants and spontaneous mutants were isolated that could utilize xylose as a sole carbon source but could not produce sufficient acid to convert bromcresol purple to its yellow form. On the other hand all

strains that were able to convert bromcresol purple to its yellow form could utilize xylose as an electron donor for the nitrite reduction and conversely those which could not produce detectable acid could not use xylose as an electron donor to reduce nitrite either. Tsukamura and Mizuno (1968) showed that being able to utilize a carbohydrate as a sole carbon source does not imply being able to produce acid from it. Although not discussed by these authors, this is due to the fact that certain modes of oxidative metabolism do not necessarily result in the accumulation of acidic products.

C. *Development of the selective minimal medium.* The selective minimal medium employed in these studies was modified during the course of the experiments for three reasons. Firstly, it was learned that the addition of Fe^{+++} enhanced growth of *M. phlei*. The reason for this is not known, but one may speculate that there is some relationship between Fe^{+++} enhancement of growth and the formation of mycobactins, iron-chelating substances produced by many mycobacteria including *M. phlei*, which permit growth of *M. paratuberculosis* (Snow, 1970). Secondly, by employing strains with two genetic markers it was possible to utilize a selective medium that would not permit the growth of either of the parent strains except for spontaneous mutants. The xylose minimal medium supplemented with streptomycin fulfilled these prerequisites. By using this medium it was possible to eliminate the possibility of the donor strain being inadvertently transferred in the phage lysate, presumably as an L-form or other cell wall deficient form that might not be evident by routine tests for bacterial sterility. Cell wall deficient forms have been demonstrated in mycobacteria (Mattman et al., 1960). Thirdly, the results of the first transduction experiment showed that while the ability to utilize xylose

as a sole carbon source could be transferred, the ability to produce sufficient acid from xylose to convert bromcresol purple to its yellow form, could not. Therefore, the bromcresol purple was omitted in the final form of the selective minimal medium, MM'.

D. *Genetic transfer in M. phlei.* The results of the early genetic transfer experiments in which the phage was not pretreated with DNase revealed that autoclaving which destroyed all plaque forming activity of the phage, did not eliminate all transfer activity (Tables 7 and 8). Furthermore it was evident that the transfer activity of the phage preparation was influenced by treatment with DNase while plaque forming activity was not (Table 7). This implied that transforming DNA, either from the donor bacterium or conceivably from the phage, was involved. Repeated attempts in our and other laboratories to extract transforming DNA by classical methods, involving disruption of the cell wall either by use of enzymes or mechanical disintegration, failed. However, it was possible to obtain DNA which could be characterized by CsCl buoyant density. Nevertheless, in order to provide definitive proof that the residual transfer activity present in heat killed phage preparations of experiments was transforming DNA, it was necessary to obtain transforming DNA. This aspect of the problem was solved when L. DeSalle, working in our laboratory, obtained transforming DNA from the culture filtrate of *M. phlei* SN109 by simply precipitating it with isopropanol. He further showed that DNA extracted from phage, and shown to be phage DNA by transfection, could transform recipient *M. phlei* F89 from xyl^- to xyl^+ (Juhasz et al., 1971). The phenomenon of simultaneous transduction and transformation has been previously reported for *Bacillus subtilis* and *Bacillus licheniformis* (Tyeryar et al.,

1969). However, several questions remained to be answered. Firstly, what affect did autoclaving have upon phage? Could phage DNA be released as a consequence of autoclaving? Secondly, wouldn't DNA be denatured by autoclaving or by heating to 99.5 ± .5 C as was done in later experiments? The answer to the first question became apparent when Ritchie (1970) reported that an excellent method of obtaining phage DNA was simply to heat the preparation to 60 C. He used coli phages T_1, T_2, T_3, T_4, T_7 and P22. He emphasized that Freifelder and others had previously shown essentially the same phenomenon for subtilis phages SP-8, osaka 1, and α and that in all cases the DNA was shown to be released as unbroken molecules. Therefore if autoclaving did not denature the DNA it was probably releasing it intact from the phage since heating ruptures the phage head. The second question could be answered when the GC ratio of the DNA became known. Since the GC ratio of both the donor strain *M. phlei* SN109 and the phage Bo2 is quite high, 72%, the DNA is extraordinarily heat stable. It does not denature until about 100 C in extremely low ionic strength solutions (DeSalle, 1971) and will not denature at all in solutions greater than 0.1M NaCl or other ions at 100 C. We may, therefore, conclude that in solutions of high ionic strength, e.g., HIB, especially under pressure, phage Bo2 DNA is not denatured. Evidence for transformation by heat stable DNA comes from experiments listed in Tables 7 and 9. In these experiments heat killed phage appeared to retain the transfer activity lost when the phage preparation was treated with DNase (Sum of lines 2 and 3 of Tables 7 and 9 as compared with line 1).

The fact that DNase treated phage preparations which were not autoclaved or inactivated by antiphage serum still showed the bulk of transfer activity,

indicated that transduction was also occurring. DNase treated phage preparations retained approximately 70 percent of such activity (Tables 7 and 9). Transfer was virtually eliminated by heating the phage preparation or by treating it with antiphage serum (Table 13). An interesting aspect of this system is that the recipient bacterium had to be approximately 48 hr old for successful transduction. Cultures just entering the stationary phase gave the highest number of xyl^+ colonies. This was a surprising result, since although this is common to many transformation systems, no prior reports on this phenomenon could be found for any other transduction system. The interpretation of this observation is further complicated by the fact that adsorption of Bo2·SN109 to the recipient *M. phlei* F89 occurs to approximately the same extent with a 24 hr bacterium as with a 48 hr bacterium. A possible hypothesis would be that although the phage can adsorb to the bacterium at any phase of its growth, its DNA can only penetrate the bacterial cell wall during a certain phase of cell growth. This hypothetical phage host relationship is not common but has been recently reported to exist in *Caulobacter* (Shapiro et al., 1971), where differentiation of the recipient bacterium is necessary to permit penetration of phage DNA although adsorption of the phage can occur at any stage in the life cycle of this organism. The failure to show genetic transfer in the experiment in which a 24 hr culture was resuspended in the sterile filtrate of a 48 hr culture seems to imply that no extracellular "competence factor" is involved. However, competence factors cannot be isolated even in well elaborated transformation systems (Hayes, 1968). Further evidence that the sole mechanism of transfer when the phage is pretreated with DNase, is by transduction comes from the experiments utilizing

HFT lysates. DNase has no affect on the number of xyl^+ colonies obtained (Table 18). Phage conversion, the appearance of a new character due solely to phage infection, could not have accounted for the observed phenomenon since phage Bo2 which had not been previously propagated on a xyl^+ donor failed to confer the ability to utilize xylose on a xyl^- recipient (Table 5).

The frequency of transduction, that is, the number of xyl^+ colonies per input phage, was fairly constant in all six sets of experiments in which 6×10^8 PFU/ml phage pretreated with DNase was mixed with bacteria and incubated for 60 min or less. The value of 2×10^{-7} obtained as the average for frequency of transduction using DNase treated phage preparations is lower than that obtained in some other transduction systems, but compares closely with that obtained for the system in *M. smegmatis* (Sundar Raj and Ramakrishnan, 1970). All of these calculations were made utilizing data corrected for spontaneous mutation obtained in each experiment. Problems were encountered when trying to obtain viable counts of mycobacteria. The only method that gives any consistent values is plating. Viable-count estimates based on colorimetry were too inaccurate to be used in these experiments. The reason for this difficulty is the natural clumping of individual mycobacteria. This can sometimes be inhibited to some extent by the use of Tween-80, a surface active detergent. However since Tween also prevents adsorption of phage its use was prohibited in transduction experiments. On the other hand, Tween-80 was used to prevent clumping during single step growth studies of the phage.

E. *Characterization of xyl^+ colonies.* Because both the donor and recipient strains were thoroughly characterized before the transduction experiments

were performed, it was easy to establish the identity of the transductants and spontaneous mutants as *M. phlei* and to show that these colonies were not the donor or recipient strains. The parent strain SN109 could utilize xylose as a sole carbon source and could produce acid from xylose. The recipient strain F89 could not utilize xylose as a sole carbon source and could not produce detectable acid from xylose. In addition, the recipient strain and the xyl^+ colonies obtained were streptomycin resistant in all experiments subsequent to the first one. The xyl^+ colonies obtained in these experiments could utilize xylose as a sole carbon source but could not produce detectable quantities of acid from xylose. The transductants could be shown to have acquired a stable hereditary character since after serial passages on complex nutrient media and subsequent washings 80% of the colonies obtained in experiments utilizing viable phage could still utilize xylose as a sole carbon source. In contrast only about 40% of the colonies obtained in experiments involving heat killed phage and even less in experiments with no phage, retained this character after several washings. Of notable interest is the fact that microcolonies tested did not retain the ability to utilize xylose. One may speculate about possible explanations for their origin: Firstly, they could be abortive transductants which are incapable of passing the acquired genetic information to their progeny (Hayes, 1968); secondly, they may be satellite colonies growing as a result of byproducts formed by actual xyl^+ colonies; and thirdly, they could be artifacts caused by the clumping phenomenon, presumably growing on contaminating nutrients entrapped within the clumps. No matter what their origin was due to, the finding that they did not retain the ability to utilize xylose justified the decision not to consider

these as xyl^+ colonies at final counting.

The discovery that seven out of the eight xyl^+ transductants were lysogenic by classical criteria, i.e., they produced phage and were immune to superinfection by the phage with which they were infected (Adams, 1959), led to the development of the HFT lysate. No explanation can be given as to why only four colonies produced as much as about 10^4 PFU/ml of phage.

F. *High frequency transduction.* High frequency transduction in mycobacteria has not been previously reported. The highest frequency obtained, about 1×10^{-2}, is higher than that reported for *Bacillus* phages but is lower than that reported for coliphages λgal and λbio (Ozeki and Ikeda, 1968). The relationship between the ability to remain lysogenic after growth in antiphage serum and the capacity to produce an HFT lysate poses an interesting question. Are we dealing with actual lysogeny, as exemplified by the coliphage λ system, or are we dealing with a carrier state, as exemplified by *Bacillus subtilis* and subtilis phage SP10 systems (Bott and Strauss, 1965)? The results seem to indicate that both are possible in the *M. phlei* F89-Bo2 system. In the case of "true" lysogeny, where the bacterium cannot be cured by growth in antiphage serum, particles can arise with the property of HFT, but not in the case of autonomous phage multiplication when antiphage serum can prevent reinfection of susceptible segregants by phage (Hayes, 1968). The finding that frequency in high frequency transduction is not constant but varies from experiment to experiment indicates that there is no direct correlation between the number of HFT particles and the number of plaque forming particles. One may speculate that the HFT particle in our system is probably defective as is the HFT particle of coliphage λ. If the mycobacteriophage does contain bacterial

genome in place of phage genome it probably cannot function as a lytic phage. One could also speculate that if lysogeny is a transitory phenomenon (Arber, 1963) the change in HFT frequency is a manifestation of the unstable relationship of phage and bacterium within each lysogenic population. This could also explain the apparent decrease in HFT capability when the HFT lysogens are stored at 4 C.

The high frequency transduction system is not affected by DNase, but requires the recipient bacterial culture be 48 hr old, indicating that transfer occurs solely by transduction in HFT. This confirms the importance of a certain growth phase for transduction to occur. High frequency transduction experiments gave the best results when very low MOIs were employed. This was in contrast to the results of low frequency transduction experiments which gave the best results when the MOI was about 1. The reason for this discrepancy becomes evident when the spontaneous mutation rate is taken into consideration. When low MOIs were used in the low frequency transduction experiments the number of spontaneous mutants were greater than the number of transductants. On the other hand, in cases where low multiplicity could be employed, adsorption of the phage was greater which allowed for more efficient transduction. This explains why the high frequency transduction worked best at low MOI. In HFT experiments the frequency of spontaneous mutation was so low as to be considered almost negligible when compared to the frequency of HFT; about 6×10^{-8} for the former as compared to about 5×10^{-3} for the latter.

G. *Characterization of high frequency transductants.* Transductant colonies from HFT experiments were larger and appeared more rapidly than those from

LFT experiments. This could be explained by the finding that these bacteria were not lysogenic. Juhasz (1968) has shown that lysogenization of *M. phlei* F89 with phage Bo2 can greatly retard its growth.

H. *Characterization of phage Bo2.* Phage Bo2 forms hazy plaques. This is due to the number of bacteria which survive phage lysis. It is this specific property which seemed to indicate that phage Bo2 would be a suitable candidate for the mediation of genetic transfer. The major drawback was that the highest titer obtainable was about 6×10^9 PFU/ml, despite many passages on its host. The phage titer could not be increased by centrifugation due to extreme loss of viability. This is probably due to the fragile nature of the phage, a property not uncommon to flexible-tailed phages (Bradley, 1967).

The electron micrographs included in this study show the size, shape and flexible nature of the tail, as well as the knob like appearance of the end of the tail. Kölbel's (1971) study on mycobacteriophages reveals the striated nature of the phage tail and more clearly outlines the hexagonal symmetry of the phage head.

The GC ratio obtained for *M. phlei* is within the range of other values reported by other workers. The range of values is from 63% to 73% GC (Bradley, 1970; Mandel et al., 1970; Marmur and Doty, 1959; Schildkraut et al., 1962; Tarnok, Röhrscheidt, and Bönicke, 1967; Wayne and Gross, 1968; Sellers and Tokunaga, 1966). Sellers and Tokunaga (1966) studied *M. phlei* F89 the recipient strain used in this study. They obtained a value of 68% GC from CsCl buoyant density. However they also reported that it banded coincident with *Micrococcus lysodeikticus* DNA but erroneously cited its value at 68%, quoting Marmur and Doty (1959). Marmur and Doty actually gave the

the value of 72% GC for *M. lysodeikticus* and this agrees precisely with the value obtained in this study. J. Kraiss, in our laboratory, confirmed that the DNA was double stranded by hyperchromicity tests and also obtained a 72% GC value for *M. phlei* F89 and *M. phlei* SN109 as well as for phage Bo2 (Kraiss, 1972; and Gelbart, Juhasz and Kraiss, 1972).

In order to establish host range, both undiluted and diluted phage suspensions should be used on a variety of hosts. Non-specific lysis can occur when high concentrations of phage are used in testing. Upon further dilution zones of non-specific lysis do not form single plaques. It was for this reason that all strains first found to be susceptible to 10^7 PFU/ml (Table 23) were diluted to single plaques to find the relative efficiency of plating (Table 24). The RTD (routine test dilution), i.e., the highest dilution of a phage suspension still producing confluent lysis, is now utilized by workers who are attempting to phage-type mycobacteria (Redmond, 1963).

The polyvalent host range of Bo2 was surprising since, although polyvalent upon initial isolation, it could be rendered specific for *M. phlei* upon serial passage on *M. phlei* strain F89 (Juhasz and Bönicke, 1965). James Kraiss and I decided that we must clarify why the phage Bo2 with which we were working had a different host range than that described in an earlier report by Juhasz and Bönicke (1965). The only difference in the treatment of the phage was that it was propagated on a different host. We obtained the originally described phage from Borstel and propagated one half of the suspension on strain F89 and the other half on strain SN109. The finding that Bo2 propagated on strain F89 only lysed *M. phlei* strains confirmed the report of

Juhasz and Bönicke (1965) but Bo2 propagated on strain SN109 was found to be capable of lysing several *M. smegmatis* strains (Table 25). We can offer no explanation as to why the host range of phage Bo2 is broadened by passage through strain SN109. Our studies also showed that the original host range cannot be restored by passage on strain F89.

The single step growth curve obtained in this study is comparable to those reported by others working with mycobacteriophages. Bowman (1958) reported a minimum latent period of 240 minutes in a study of several mycobacteriophages. In a later paper (Bowman and Redmond, 1959) he reported that phage D29S had a latent period of 90 minutes with a burst size of 18. Bowman explained the low burst size and the apparent stepwise growth curve in the following manner: "Several preliminary one step growth experiments (Adams, 1959) did not give the classical one step growth results: A sharp leveling or plateau (defining the single bursts) did not appear; instead, a gradual two-step or three-step growth occurred. Cycling is usually prevented by the high dilution from the adsorption tube to the first growth tube. Apparently cycling occurred independently of (1) the low adsorption rate constant for D29S on *Mycobacterium ranae* and (2) the high dilution made from adsorption tube to first growth tube. A consideration of the clumped state of the bacteria in the first growth tube led to the hypothesis that adsorption of new phage progeny could occur independently of the two factors mentioned above because phage progeny from a cell contained within a bacterial clump would only have to diffuse over relatively short distances to infect adjacent bacteria". Phage Bo2 also shows poor adsorption and Bowman's hypothesis could be one explanation for the observed low burst size. There can be no question

that clumping certainly is a complicating factor in any quantitative study of average burst, e.g., in a single step growth curve, or single burst as was performed during the present study. Thorough mixing of the cultures on a vortex type mixer was employed during all phases of the study to keep the clumps of bacteria as small as possible. However, microscopic examination revealed that it was impossible to prevent clumping and that the size of the clumps increased with time, even with shaking. The finding that not all infective centers produced bursts could also be attributed to the fact that we were dealing with clumps of bacteria in each colony forming unit. However, there is also the possibility that a large proportion of the phage population enters into a symbiotic state with their host bacterium which may have resulted in lysogeny. This relationship certainly requires further investigation.

I. *The failure to transfer additional markers.* Until now one marker, the ability to utilize xylose as a sole carbon source, could be transferred by phage Bo2. All attempts to transfer drug resistance markers have failed. Although auxotrophs are currently being developed by the use of nitrosoguanidine (Holland and Ratledge, 1971) at the present time no mutants that could be considered useful as recipient strains for transfer experiments have as yet been isolated. For this reason it is impossible to say with certainty at this time whether we are dealing with a specialized or generalized type of transduction. All accumulated evidence seems to indicate that this is probably a case of specialized transduction: 1) the low initial transduction frequency; 2) the failure to transfer additional markers; 3) the isolation of HFT lysates; and 4) the finding that transductants which produce HFT

particles appear to have phage integrated into their bacterial chromosome, since they cannot be cured by growth in antiphage serum.

J. *Summary and conclusion.* A transduction system in *Mycobacterium phlei* has been developed and characterized. It is clearly shown to be transduction because all other genetic transfer mechanisms can be discounted: 1) donor bacteria can be removed by filtration thus eliminating conjugation as a possible mechanism; 2) phage pretreated with DNase retain transfer activity which is not affected by DNase, thus eliminating transformation by free DNA; and 3) phages not propagated on the donor possess no transfer activity, thus eliminating phage conversion. On the other hand evidence which supports the conclusion that transduction is the mechanism of transfer is: 1) transfer activity is proportional to phage concentration at a given multiplicity of infection; 2) transfer activity of phage pretreated with DNase is lost upon treatment with antiphage serum or upon heating; and 3) DNA extracted from phage Bo2 propagated on the donor strain is capable of transformation by transfection (DeSalle, 1971).

The development of a transduction system in the genus *Mycobacterium* can be viewed as a significant step toward establishing a useful genetic transfer system in this genus and furthermore understanding the specifics of this transduction system can only help us to better understand the phage-host interrelationship in mycobacteria. However, as with any scientific study, questions have arisen which remain to be answered. For example, why do the recipient bacteria have to be approximately 48 hr old for transduction to occur? This and similar questions could not have been asked until this study was undertaken. For this reason, it is the opinion of the author that this

study has not only answered the question, whether transduction exists in *Mycobacterium*, which was clearly the main purpose of the project, but it has opened up new avenues in the study of phage-host relationships in mycobacteria.

SUMMARY

Transduction, the transfer of genetic information from one bacterial host to another by bacteriophage, has been demonstrated to occur in *Mycobacterium phlei*. The ability to utilize D-xylose as a sole carbon source (xyl^+) could be transferred from *M. phlei* strain SN109 (xyl^+, str-s) to *M. phlei* strain F89 (xyl^-, str-r) by a bacterially sterile, DNase treated phage Bo2-lysate of donor *M. phlei* SN109. The marker was expressed within 15 minutes of phage infection, but the recipient bacteria had to be in early stationary phase for transduction to occur. Controls employed in these experiments were bacteria incubated with heat killed phage, with phage plus antiphage serum, or with sterile broth in place of phage. Phage conversion, the appearance of a new character due solely to phage infection, could not account for the observed phenomenon since phage Bo2, which was not previously propagated on a xyl^+ donor, failed to confer the ability to utilize xylose on a xyl^- recipient.

A high frequency transducing filtrate could be obtained from xyl^+ transductants without induction. This filtrate, after treatment with DNase, could transfer the xyl^+ marker with a rate approximately 10^4 greater than that of the original phage preparation, which was about 2×10^{-7}.

Although attempts to transfer other characters have failed, it is still not possible to ascertain with certainty whether the transduction by Bo2·SN109 is of the generalized or specialized type. However, evidence of phage integration in lysogenic transductants strongly suggests that this is specialized transduction.

The transducing phage Bo2 has been studied with respect to several major

characters. It forms hazy circular plaques 3-5 mm in diameter. The nucleic acid is double stranded DNA with a GC ratio of 72%. It adsorbs very poorly to its host bacterium. The one step growth characteristics are: a latent period of about 4 hr; a rise period of approximately 3 hr; and a burst size of approximately 10. Single burst experiments verified the low average burst and showed that not all infected bacteria produce bursts even after 8 hr.

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APPROVAL SHEET

The dissertation submitted by Sheldon Mark Gelbart has been read and approved by the undersigned faculty members.

The final copies have been examined by members of the Dissertation Committee and the signatures which appear below verify the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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