CHROMOSOMAL LOCATION FOR A GENETIC DETERMINANT OF CHLORTETRACYCLINE RESISTANCE IN A STRAIN OF STAPHYLOCOCCUS AUREUS

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ABSTRACT

Chromosomal Location For A Genetic Determinant Of Chlortetracycline Resistance In A Strain Of *Staphylococcus Aureus*

by Doris Beck

The genetic determinant for chlortetracycline resistance in strain U9 of *Staphylococcus aureus* was found to have both chromosomal and extrachromosomal nature. Strain U9 was also resistant to both erythromycin and streptomycin which were studied for comparison to the tetracycline resistance.

Chlortetracycline resistance was apparently stable in strain U9 because no sensitive colonies were detected during a series of curing experiments which utilized growth in solutions of acriflavine, ethidium bromide, and also growth at elevated temperatures. Low doses of ultraviolet radiation on transducing phage lysate propagated on strain U9 caused an increase in transduction frequency for all three antibiotic resistances.

The transduction frequency for chlortetracycline resistance with phage lysate propagated on U9 was more than a log higher than for the other chromosomal resistances. This transduction frequency was higher than that for chlortetracycline resistance obtained when phage 80 was propagated on

strain 2 (pen, tet), an example in which the antibiotic resistances are known to be plasmid linked. Transduction frequencies for extrachromosomal markers are generally higher than for those of chromosomal markers because extrachromosomal material does not depend on recombination within the host for gene expression.

Transductants obtained with nonirradiated phage propagated on U9 were not as stable as those obtained with irradiated phage because sensitive colonies were found in curing experiments. Three sensitive colonies were isolated with a concommitant loss of penicillinase production. Three additional colonies of strain 2 (pen, tet) also showed this coelimination.

Chlortetracycline resistance may be due to a heterodiploid state in U9 as both chromosomal and extrachromosomal properties were observed. An alternative explanation could be that a resident plasmid within the transductants interacted with the chromosome extracting the tetracycline resistance which yielded variants with plasmid linked resistance.

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INTRODUCTION

Since the advent of antibiotic therapy many of the enterococci and staphylococci have developed resistance to various antibiotics (Barber, 1966). The rapidity in which high levels of resistance are attained and the widespread occurrence of such resistances invoke more than merely the operation of evolutionary processes. Mutations occurring *in vitro* are observed to consist generally of slight increases in resistance requiring a long time to achieve any high level of drug resistance (Florey, 1949).

Watanabe (1961) reports that high level multiple drug resistance in a strain of *Shigella flexneri* can be transferred via episomes to strains of *Escherichia coli*, *Salmonella typhimurium*, and *Salmonella enteritidis*. A conjugal relationship is not observed in staphylococci and thus the only means for transmitting resistances between strains appears to be by bacteriophage. Jarolmen et al. (1965) succeeded in transducing tetracycline resistance into sensitive strains of *Staphylococcus aureus in vivo* by injecting infected mice with phage propagated on a tetracycline resistant strain of *Staphylococcus aureus*.

The mechanism for tetracycline resistance varies with the particular resistance transfer factor within strains of Escherichia coli (Unowsky and Rachmeler, 1966) or with the nature of the resistance determinants, ie., whether they are of chromosomal or extrachromosomal origin (Weisblum and Davies, 1968). Asheshov (1966) and Poston (1966) have demonstrated that tetracycline resistance is extrachromosomal in certain curable strains of Staphylococcus aureus. The purpose of this study is to determine whether the genetic locus for chlortetracycline resistance in strain U9 of Staphylococcus aureus is located on chromosomal or extrachromosomal DNA and to observe any relationships or interactions of chromosomal and extrachromosomal markers.

HISTORICAL REVIEW

Chromosome mapping in Staphylococcus aureus

Chromosome mapping of Staphylococcus aureus has been limited due to the absence of sexuality in this organism. Altenbern (1969) has mapped some markers on the chromosome of Staphylococcus aureus by using phenethyl alcohol to synchronize chromosomal replication and then subjecting the bacteria to treatment with N-methyl-N'-nitro-N-nitrosoguanidine at various times after resumption of DNA synthesis. His results indicate that there is a definite locus on the chromosome for the initiation of chromosomal replication and that the gene order is essentially the same in randomly selected strains of Staphylococcus aureus.

Altenbern reports that strains containing multiple-resistance factors (MRF) show a delay in the gene replication time possibly because the MRF duplicates first after which chromosomal replication is permitted.

Plasmids in staphylococci

The type of plasmid found in the staphylococci are nontransmissible plasmids which are manifest by the establishment of drug resistance in the host. It appears that plasmids are dispensable for the maintenance of cell viability but are genetically stable with some possible

exceptions. Novick (1963) has found the spontaneous rate of loss for the penicillinase plasmid to be about 10^{-3} per cell generation.

Evidence for plasmid linkage of somatic traits

Novick (1969) gives a list of criteria for determining plasmid linkage. Coelimination studies such as curing by growth in solutions containing acriflavine (Mitsuhashi et al., 1963), ethidium bromide (Bouanchaud et al., 1969), or rifampicin (Johnston and Richmond, 1970), and growth at elevated temperatures provide evidence for the autonomous nature of plasmids and those somatic traits carried on them.

Irradiation of phage lysate utilized in transduction reveals the extent of genetic homology of markers to the host chromosome by the ability of the chromosome to rescue these markers (Garen and Zinder, 1955 and Arber, 1960). Low doses of ultraviolet irradiation produce an increase in transduction frequency for markers carried by DNA homologous to the host chromosome and thus provide a means of differentiating between episomal and chromosomal locations of markers. Plasmid DNA is not homologous to the chromosome and as increasing dosages of irradiation damage the plasmid carried by the transducing phage the less likely the survival of the plasmid and its somatic characteristics.

Replicative autonomy of somatic traits may be demonstrated by radiation target size as the plasmid is much

larger than a chromosomal gene. Incompatibility with a known plasmid constitutes evidence of plasmid linkage (Novick and Richmond, 1965). Plasmids belonging to the same compatibility group can not exist as stable diploids within a cell so that the establishment of the incoming plasmid requires the loss of the resident plasmid. Rush et al. (1969) have demonstrated the autonomy of staphylococcal plasmids by isolating the plasmid DNA and preparing electron micrographs of the DNA by autoradiography. Their isolating of plasmid DNA was based on the resistance to alkali denaturation of the covalently closed circular duplex DNA.

Current genetic aspects of antibiotic resistances

The incidence of drug resistant staphylococci has been extensively investigated by Barber (1966) who revealed the increasing numbers of resistant strains in clinical infections since the beginning of antibiotic therapy. She asserts that staphylococci develop resistance to erythromycin and streptomycin more readily than to most antibiotics. This ability would appear to reflect the chromosomal locus for these antibiotic resistances as shown for streptomycin by Korman and Berman (1962) and Poston (1966) and for erythromycin by Richmond (1969). Mutations occurring in bacteria are most likely changes

of the chromosomal DNA as the chromosomal DNA is many times the plasmid DNA and because the chromosome is an essential feature of all cells.

In contrast the extrachromosomal nature of erythromycin resistance has been demonstrated by Mitsuhashi et al. (1965) by joint elimination of penicillinase production and erythromycin resistance utilizing ultraviolet irradiation and growth in acridine. May et al. (1964) and Asheshov (1966) have shown that loss of tetracycline resistance can be accelerated by growth at elevated temperatures illustrating an extrachromosomal inheritance in some strains. Asheshov (1966) and Foston (1966) utilized irradiation inactivation kinetics of transducing phage lysates to demonstrate the plasmid linkage for tetracycline resistance and the chromosomal location for penicillinase production in some strains of Staphylococcus aureus. The strains used by these investigators had unstable resistance to tetracyclines but stable determinants for producing penicillinase.

Asheshov (1969) in reference to penicillinase production and Richmond (1969) for erythromycin resistance refer to the transition of these genes between integrated chromosomal loci and the extrachromosomal state. These investigators are finding that resistance to some drugs may show both chromosomal and extrachromosomal

inheritance within a strain of staphylococci at different periods of time. Richmond (1969) demonstrates that there is a reversible transition between the extrachromosomal and the integrated state for erythromycin resistance in some strains of Staphylococcus aureus.

Mechanism of tetracycline activity

The tetracyclines are a group of antibiotics composed of four fused rings including the following: aureomycin (7-chlortetracycline), terramycin (5-hydroxy-tetracycline), and declomycin (demethylchlortetracycline). The microbial spectra and biochemical activities for these antibiotics are essentially the same so that effects using one antibiotic usually are paralleled by the others. Chlortetracycline is somewhat unstable in liquid media so that determinations of resistance levels for strains of bacteria are overestimated.

The tetracyclines are inhibitors of protein synthesis that effect the ribosome (Weisblum and Davis, 1968). Tetracycline has been shown by Day (1966) to bind to both the 50 S and 30 S subunits of the ribosome. Suarez (1965) has reported that tetracycline inhibits the binding of aminoacyl-tRNA to 30 S subunits in vitro and by this mechanism perhaps inhibits polypeptide synthesis. Day (1966) has found that if either the 50 S or 30 S subunits are treated with tetracycline, washed by dialysis and centrifugations, and then studied in cell free extracts of Escherichia coli that protein synthesis is inhibited and that the tetracycline

molecules are distributed afterwards between both subunits. Gurgo et al. (1969) have studied the synthesis and location of mRNA in cells treated with tetracyclines and other inhibitors of protein synthesis. They report that peptide bond formation and polyribosome formation are "uncoupled" by the antibiotics. Their observation that newly formed mRNA continually appeared in polyribosomes suggests that the ribosomes must add to and move along the mRNA without the occurrence of protein synthesis. In any event the site of action for tetracycline has not been localized and the reaction of the tetracyclines may be more extensive than binding to the ribosomes.

Resistance to tetracyclines may be due to a permeability factor or other protective barriers such as drug inactivation depending upon the organism and the genetic determinant for resistance as reported by Unowsky and Rachmeler (1966). They were able to show reduced uptake of tetracycline by Escherichia coli containing the negative fertility inhibition episome but not for those cells containing the positive fertility inhibition episome. Unowsky and Rachmeler were unable to isolate an enzyme active on tetracycline for Escherichia coli containing the positive fertility inhibition resistance-transfer (R) factor.

MATERIALS AND METHODS

Media

Cultivation of organisms. Bacto Brain Heart Infusion (BHI), BHI agar, nutrient broth, and nutrient agar (Difco Laboratories, Detroit, Mich.) were used in routine cultivation of bacteria. For phage propagation nutrient agar and nutrient broth were enriched with dextrose and phosphate (K_2HPO_4), described and named P and D media by Pattee and Baldwin, 1961 (cf. pp 17).

Selective media. Chlortetracycline hydrochloride and erythromycin (Sigma Chemical Co., St. Louis, Mo.) were sterilized with a membrane filter (Millipore Filter Corporation, Bedford, Mass.) having a pore diameter of 0.45 u. The solutions of antibiotics were frozen and stored at -20 C until needed and then added to sterile tempered (molten agar between 45-50 C) media before pouring plates.

Dihydrostreptomycin sulfate (E. R. Squibb & Sons, Div. of Olin Mathieson Chemical Corp., New York, N. Y.) and Kantrex (Bristol Laboratories, Div. of Bristol-Myers Co., Syracuse, N. Y.) were purchased sterilized so that sterile solutions of these antibiotics were simply added to hot sterile BHI agar.

Curing agents. The curing agents used were Ethidium bromide, 2,7,-diamino-D-ethyl-9-phenylphenantridium bromide

(Calbiochem in Los Angeles, Calif.) and Acriflavine HCl, a mixture of the hydrochlorides of 2,8(3,6) Diamino-D-meth acridinium chloride and 2,8(3,6) Diaminoacridine (Sigma Chemical Co. in St. Louis, Mo.). Stock solutions containing 5000 ug/ml were sterilized by membrane filtration as described previously and stored at 4 C. The curing agents were added to BHI broth to achieve desired concentrations.

Strains of Staphylococcus aureus and phages

Identification and sources. Strains U9(ery, pen, str,tet) and 8325 (str, tet) were supplied by Dr. Richard P. Novick of the Public Health Research Institute of the City of New York, Inc., New York, N. Y. Dr. M. H. Richmond of Bristol University Medical School in Bristol sent strain 147(str). Dr. E. H. Asheshov of the Central Public Health Laboratory in London kindly submitted a culture of strain 2(pen, tet). Strain PS55 and A3, propagating strains for phages of the International Typing Series came from our stock. Phage propagating strains 53 and 80 along with phages 53 and 80 of the International Typing Series were donated by the Michigan State Public Health Department. Strains SH(1-5) are clinical isolates that were received from Edward W. Sparrow Hospital in Lansing, Mich. Strains A-G are tetracycline resistant transductants of strain 80 obtained using five minute ultraviolet irradiated phage 80

propagated on strain U9. Strains 1-8 are tetracycline resistant transductants of strain SH5 obtained with nonirradiated phage 80 propagated on strain U9.

Nomenclature. Strains of Staphylococcus aureus have been identified by using the first three letters of the antibiotics to which the strain is resistant following the strain number.

Phage typing. Sensitivities and/or resistances to phage 80 and 53 were determined for most strains utilized. When needed, relationship of strains was ascertained by a complete typing carried out by the Michigan State Public Health Department in Lansing, Mich.

Cultures to be phage typed were cultivated on BHI agar slants containing 7.5% NaCl for 16 to 18 hours at 37 C. The bacteria were suspended in 5 ml of P and D broth and diluted so that a 0.1 ml sample contained approximately 5×10^3 cells. Cell concentrations were determined by measuring absorbance with a Bausch & Lomb Spectronic 20. Tempered soft P and D agar (2.5 ml) was inoculated with 5×10^8 bacteria and poured over a P and D base agar. The plates were allowed to dry and then spotted with 0.1 ml of typing phage. The plates were incubated at 37 C and checked for lysis after 3, 16, and 24 hour periods. This procedure is a modification of the method used by Pattee and Baldwin (1961).

Determination of drug resistance

Disc assay. Bacto sensitivity discs for antibiotics (Difco) were used to assay for resistance to antibiotics. Inorganic assay discs were made using discs (6.35 mm in diameter manufactured by Carl Schleicher & Schuell Co., Keene, N. H.) which were sterilized by autoclaving for 25 minutes at 20 pounds pressure, dried, and impregnated with 0.01 ml of inorganic ion solutions sterilized previously by autoclaving. The amounts of inhibitors impregnated on discs are listed in Table 1. The amounts of inorganic ions were the concentrations found to be most suitable in demonstrating variability of staphylococci by Novick and Roth (1968).

Four inhibitor impregnated-discs were placed on BHI agar plates seeded with 10^8 cells of the strain to be tested. The plates were subsequently incubated at 37 C for 16 to 24 hours. Resistances and/or sensitivities were determined by measuring the radius of the inhibition zone around the disc.

Graded plate assay. A series of graded plates was made up by adding suitable dilutions of the antibiotic to BHI agar. An overnight BHI culture of the organism to be tested was spotted with an inoculating loop on the prepared set of graded plates. Overnight broth cultures were also suitably diluted so that about 200 cfu (colony forming units) would be obtained when 0.1 ml was spread over the surface of each plate. The level of resistance was recorded as the highest concentration of antibiotic

Table 1. Preparation of assay discs for drug resistance.

Inhibitor	Amount of inhibitor impregnated per disc
	ug
Kanamycin sulfate	30.00
Streptomycin	10.00
Penicillin	10.00
Erythromycin	15.00
Novobiocin	30.00
Neomycin	30.00
Tetracycline	30.00
Chloromycetin	30.00
Sodium arsenite	13.00
Sodium orthoarsenate	3.10
Lead nitrate	3.30
Sodium metasilicate	12.20
Zinc nitrate	0.45
Cadmium nitrate	0.03
Mercuric nitrate	0.05

in which isolated colonies were able to grow or that highest concentration of antibiotic in which growth was not impaired for spotted cultures. Any effect of inoculum size was observed and noted.

Selection of drug resistant variants by gradient plates

The method of Szylbalski and Bryan (1952) for gradient plate construction was utilized to create a gradient of antibiotic concentration across a petri dish. BHI agar was used as the slanted base agar and a top agar made up of BHI agar containing antibiotic which was added after solidification of the slanted base layer. For selection of chlortetracycline resistant colonies the top agar contained 3 ug/ml chlortetracycline. Selection for kanamycin resistant organisms utilized kanamycin gradients of increasing concentration employing concentrations of 3, 10, 20, 30, and 40 ug/ml kanamycin sulfate in the top agar.

By means of a sterile glass rod duplicate gradient plates were spread with 10^7 sensitive organisms of an overnight BHI broth culture and then incubated at 37 C for 16 to 24 hours. Colonies growing in the highest concentration of antibiotic were selected, inoculated into BHI broth, and incubated at 37 C for 16 to 24 hours. The overnight broth cultures were assayed for level of drug resistance by culturing on a series of graded plates and also inoculated onto another

set of gradient plates of the same or higher concentration of antibiotic as in the previous gradient. Selection and cultivation of most resistant colonies was repeated with other passages through gradient plates to obtain drug resistant strains. Levels of resistance were measured and noted after passage through each gradient.

Curing strains of antibiotic resistance

Growth at elevated temperatures. This curing procedure was slightly modified from the method used by Ashes-hov, 1966. Resistant colonies were selected from BHI agar plates containing 15 ug/ml chlortetracycline which had been incubated at room temperature for three days previous to use, inoculated into BHI broth, and incubated at 37 C for 16 to 24 hours. The overnight cultures were diluted 1:4 in fresh BHI broth and incubated 1½ hours at 37 C in a gyrotory water bath shaking 123 cycles/min. Samples (0.15 ml) of this log phase culture were inoculated into 20 ml of BHI broth prewarmed to 37 and 43 C and incubated 5½ hours in water baths of 37 and 42.5 to 43.5 C respectively without shaking. After the incubation 5 ml samples of the cultures were shaken vigorously with 3 ml of ballotini in a vortex blendor to separate clumps of cells into single cell units. The cultures were then diluted so that 50 to 150 cfu in 0.1 ml samples were spread onto nutrient agar plates (dried

previously for three days at room temperature. The plates were incubated overnight at 37 C and replicated onto nutrient agar containing 15 ug/ml chlortetracycline. To diminish spreading of colonies all plates to be used in the replicating process were routinely dried by incubating at room temperature three days and additionally at 37 C overnight prior to use. Due to this drying period selective media is not as concentrated as indicated for chlortetracycline. Sensitive colonies which grow only on media without antibiotic were isolated and restreaked on selective tetracycline medium to reconfirm tetracycline sensitivity.

Cured colonies were compared with parent strains by disc assays and phage typing so that relationship was ascertained and linkage groups of tetracycline and other markers could be disclosed.

Growth in acriflavine. Solutions of acriflavine were made up in BHI in concentration of 25.00, 12.50, 6.25, and 3.12 ug/ml according to the method of Hashimoto et al. (1964). A colony of the resistant strain was selected from an antibiotic containing BHI agar plate, inoculated into BHI broth, and incubated at 37 C for a period of 16 to 24 hours. Samples of 0.1 ml of the overnight broth culture were then inoculated into the series of acriflavine dilutions and incubated 24 to 36 hours at 37 C. The culture of highest acriflavine concentration yielding uninhibited growth was

selected and a sample of 5 ml blended with 3 ml ballotini as previously described. Suitable dilutions were made so that 50 to 150 cfu were spread onto nutrient agar plates and incubated overnight at 37 C. Suitable plates containing isolated colonies were then replicated on nutrient agar containing 15 ug/ml chlortetracycline. Cured colonies were tested as before to confirm drug sensitivity, to ascertain relationship with parent strain, and to detect linkage with other markers.

Growth in ethidium bromide. Ethidium bromide was added to BHI broth to give final concentrations of 6, 4, 2, and 1 ug/ml (Bouanchaud et al., 1969). The experiment was performed as described for acriflavine in the preceding section.

Transduction of chlortetracycline resistance

Propagation of phage. P and D agar and P and D broth were used in phage propagation and transduction procedures following the methods of Pattee and Baldwin (1961). P and D broth is composed of nutrient broth enriched with 0.2% dextrose and 0.25% phosphate (K_2HPO_4). P and D base agar and soft agar contain 1.5% and 0.3 % agar respectively. Typing phage 80 was propagated on strain U9 (pen, str, tet) using the agar-layer technique of Swanstrom and Adams (1951).

The propagating strain was grown overnight on BHI agar slants containing 7.5% NaCl and the growth from this slant was suspended in 5 ml of P and D broth giving a suspension of approximately 2 to 6 x 10^9 cells/ml. Soft P and D agar was inoculated with 5 x 10^3 cells of the propagating strain and 10^5 to 10^6 phage. The soft agar mixture was poured over a P and D agar base, allowed to solidify, and incubated overnight at 37 C. Two ml of P and D broth were then added to each plate and the soft agar layers scraped into a sterile centrifuge tube with a sterile glass rod. The mixture was shaken vigorously to extract the phage from the soft agar and allowed to incubate 30 minutes at room temperature. The soft agar was removed from the phage lysate by centrifuging 10 minutes at 1100 rpm and collecting the supernatant lysate. To sterilize the lysate 0.5 ml of chloroform was added for each 10 ml of solution, shaken vigorously, and incubated 30 minutes at room temperature. Before use, the lysate was decanted from the chloroform layer and any remaining chloroform blown off. Titers of about 10^9 pfu were achieved by this method.

For assay, samples of 0.1 ml of suitably diluted phage were added to 2.5 ml of warm tempered soft P and D agar containing 5 x 10^8 cells of strain 80. The soft agar was poured over a P and D base agar, allowed to solidify, and incubated overnight at 37 C prior to counting plaque forming units (pfu).

Transduction procedure. Recipient strains, 30 and SH5 were grown overnight on BHI agar slants containing 7.5% NaCl and the growth from these slants was suspended in 10 ml of P and D broth giving a suspension of approximately 1 to 3 x 10^9 cells/ml. One ml of the recipient bacteria suspension was mixed with 1 ml of the phage lysate yielding a multiplicity of infection (moi) of 0.5 to 1.0. The cells were incubated in a gyrotory water bath shaker at 37 C shaking 123 cycles/min. After the incubation period the cells were diluted 1:10 to retard phage adsorption and centrifuged 10 minutes at 1100 rpm to remove unadsorbed phage. The cells were resuspended in P and D broth and assayed for antibiotic resistance by plating 0.1 to 0.2 ml samples on selective media. The three selective media consisted of BHI agar containing 1 ug/ml erythromycin, 15 ug/ml chlortetracycline, or 25 ug/ml streptomycin sulfate. Chlortetracycline plates were used three to ten days after preparation and were kept in refrigeration at 4 C until needed. BHI agar was used as it inhibits lysis of transductants by virulent phage. Controls consisting of recipient bacteria plated on selective media and sterility tests for phage plated on BHI agar were run with each transduction period. Suitable dilution and plating of recipient cells on BHI agar at time of assay was done to determine the fraction of recipient cells.

Time required for phenotypic expression. Transduced cells were resuspended in 10 ml of P and D broth after the transduction period and incubated at 37 C in the gyrotory water bath shaking 123 cycles/min. Samples of 0.1 ml were plated on chlortetracycline selective agar and 0.2 ml were plated on erythromycin and streptomycin selective agar each hour for a total assay period of 5 hours.

Ultraviolet irradiation of phage. Four ml of phage lysate suspended in P and D broth were gently swirled under an ultraviolet lamp at a distance of 40 cm at which the light intensity was 13 ergs/mm^2/sec as determined by the killing effect on E. coli K12. One ml of the radiated phage suspension was used in transduction and another ml in titering the phage. The remaining 2 ml of lysate were refrigerated at 4 C for repetition of experiment if needed. All procedures were carried out in yellow light to diminish possibilities of photoreactivation. Tetracycline transductants were assayed after resuspension of centrifuged transduced bacteria but erythromycin and streptomycin transductants were assayed after a 4 hour incubation in the gyrotory water bath since there was a phenotypic expression lag with these resistances.

Transduction frequency. Transduction frequencies were calculated as the number of transductants/input phage. The total number of transductants was calculated by

multiplying the number of resistant colonies growing on antibiotic selective media by the ratio of input recipient bacteria to the number of bacteria assayed. This method was used to normalize for multiplication of bacteria during assays or for loss of bacteria in centrifugation.

RESULTS

Phage type and drug resistance

Strains of Staphylococcus aureus were typed for susceptibilities to lytic action of phage 80 and 53. Drug resistance was determined using inhibitor disc assays and series of graded plates. Table 2 lists the results of disc assays and phage typing.

Selection for drug resistant variants

To select for kanamycin resistant mutants, cultures were passed through a series of five gradient plates of increasing kanamycin concentration and the level of resistance determined by testing the most resistant colonies isolated from each gradient on a series of graded plates containing various amounts of kanamycin.

Two subcultures of strain 8325, A3, and 147 and one subculture of U9 and PS55 were each observed in duplicate. The initial level of resistance for all strains was equal to or less than 5 ug/ml kanamycin sulfate. The levels of resistance after each passage through gradient plate selections are shown in Table 3. There was much fluctuation and variation occurring within strains so that subcultures showed a large standard deviation when results were compared. Due to this large variation in kanamycin resistance within strains it

Table 2. Phage type and chemical resistance of strains of Staphylococcus aureus.

Strain	Inorganic ion resistance	Antibiotic resistance	Susceptibility to phage[a]	
			80	53
U9	Cd, Hg, Zn.	Ery, Pen, Str, Tet	S	R
147	Cd, Hg, Zn.	Str.	S	S
8325	Pb.	Str.		
PS55				
A3				
80	Hg.	Pen.	S	R
53				S
SH1	Cd, Zn.	Ery, Str, Tet	S	R
SH2	Cd, Hg, Zn.	Ery, Pen, Str	R	R
SH3			R	R
SH4	Cd, Zn.	Chl.	R	R
SH5	Cd, Hg, Zn.	Pen.	S	R
SH6			R	R

[a]Symbols R and S denote cell resistance and sensitivity respectively to lytic action by phage. Blanks for susceptibility to phage indicate that the strain was not phage typed.

Table 3. Level of kanamycin resistance after passages through series of gradient plates with different concentrations of the antibiotic.

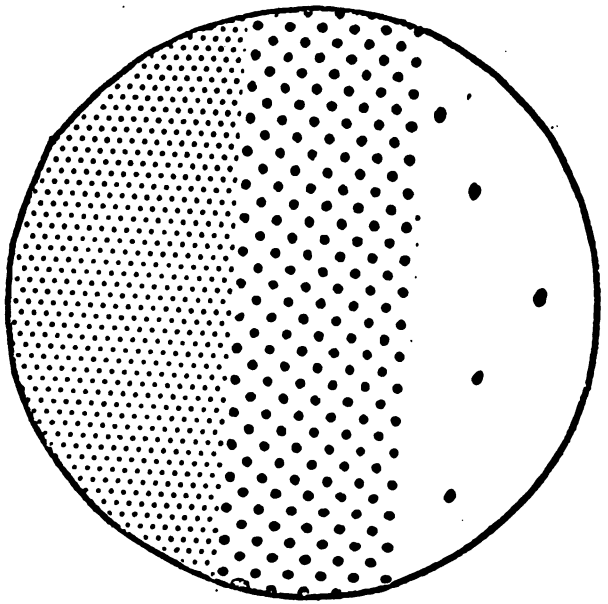
Gradient plates		Subcultures (1 or 2) of strains sensitive to kanamycin (ug/ml)											
Series	Kanamycin concentration	A3		U9		147		PS55		8325			
		1	2	1	1	2		1	1	2			
	ug/ml	ug/ml		ug/ml	ug/ml			ug/ml	ug/ml				
A	3	10 ± 0	20 ± 0	5 ± 0	7 ± 0	5 ± 0		4 ± 1	35 ± 20	5 ± 0			
B	10	10 ± 0	10 ± 0	18 ± 11	10 ± 0	25 ± 0		10 ± 0	50 ± 0	10 ± 0			
C	20	10 ± 0	20 ± 0	10 ± 0	10 ± 0	20 ± 0		10 ± 0	20 ± 0	10 ± 0			
D	30	10 ± 0	30 ± 0	18 ± 11	20 ± 14	30 ± 0		18 ± 11	30 ± 0	10 ± 0			
E	40	10 ± 0	30 ± 0	18 ± 11	27 ± 4	30 ± 0		18 ± 11	40 ± 14	10 ± 0			

was relatively easy to obtain a more resistant strain by gradient plate selection and an isolate of 147 was able to survive a concentration of 100 ug/ml kanamycin when a broth culture was spotted on a graded plate. This isolate was phage typed by the Michigan State Public Health Department ascertaining its relationship to strain 147.

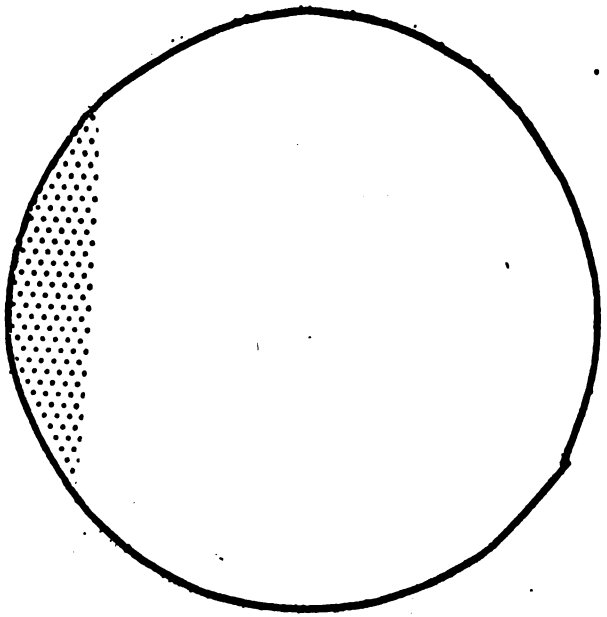
Strain A3, 147, PS55, and 8325 were similarly observed for their mutation to chlortetracycline resistance. The results are tabulated in Table 4 and show that there is little variation of resistance in these sensitive strains. Antibiotic agar used in making gradient plates contained 3 ug/ml chlortetracycline for all experiments as growth was continuously inhibited at this concentration and colony forming units were uniformly restricted to the least concentrated region of the plate. Lack of growth on gradient plates past regions of a particular antibiotic concentration or lack of variability in resistances prevented isolation of any resistant variants for chlortetracycline resistance. Two to six samples of each gradient were observed. There was a contrast of growth on the kanamycin and chlortetracycline gradient plates (Fig. 1). Fig. 2 is a graph of the average level of resistance for all strains tested versus the number of gradient plates in which the strains had been passaged.

Table 4. Level of chlortetracycline resistance after passages serially through two gradient plates containing chlortetracycline (3ug/ml)

	Strains sensitive to chlortetracycline (ug/ml)			
Gradient plate	A3	147	PS55	8325
	ug/ml	ug/ml	ug/ml	ug/ml
None	4 ± 1	3 ± 2	1 ± 2	1 ± 2
Gradient A	3 ± 2	3 ± 2	3 ± 2	3 ± 2
Gradient B	4 ± 1	4 ± 1	5 ± 2	5 ± 2



KANAMYCIN GRADIENT



CHLORTETRACYCLINE GRADIENT

Figure 1. Selected example illustrative of colony distribution on gradient plates.

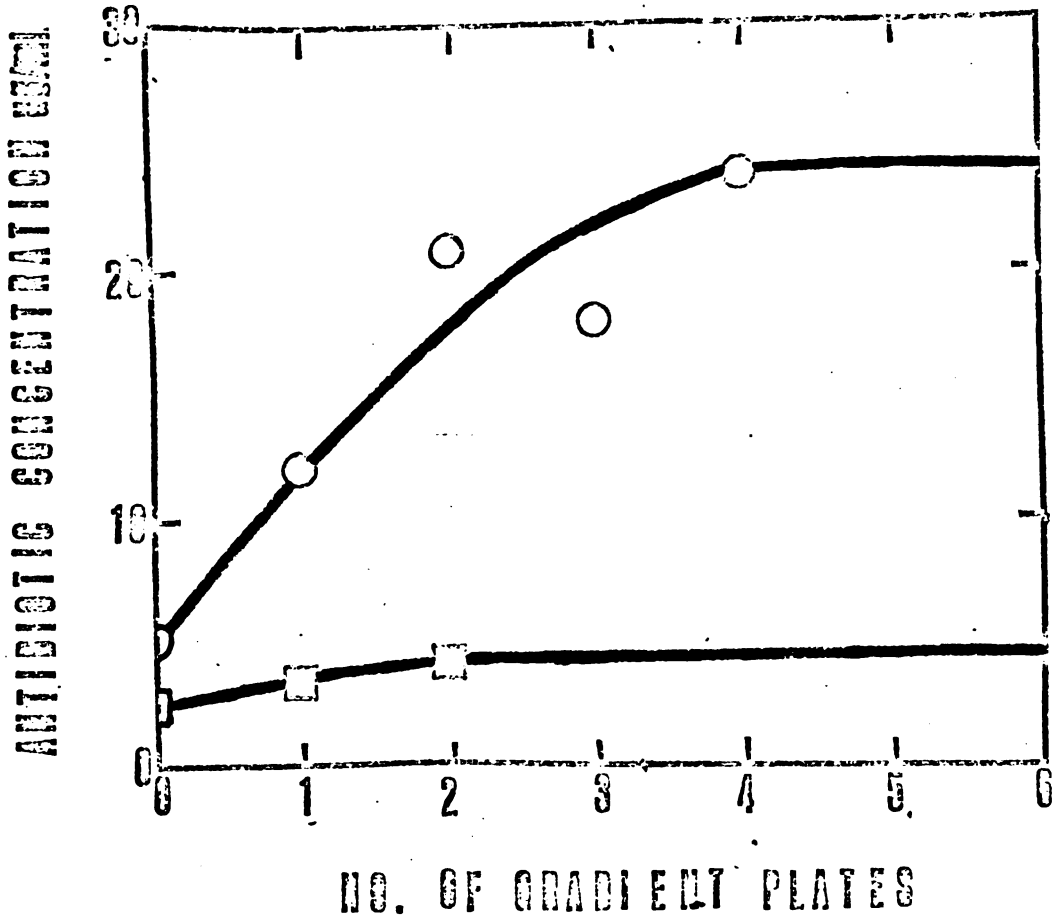


Figure 2. Effect of number of gradient plate colony selections on levels of resistance to two antibiotics. The highest concentration of antibiotic uninhibitive to growth was determined for colonies selected from gradient plates by cultivation on different graded plates of the antibiotic. Symbols: (□) level of resistance to chlortetracycline and (○) level of resistance to kanamycin.

Curing experiments

Procedures in which growth in solutions of acriflavine or ethidium bromide as well as in normal BHI broth at elevated temperatures were utilized to possibly eliminate resistance to chlortetracycline in strain U9(ery, pen, tet). The successful curing of strain 2(pen, tet) with ethidium bromide precluded using growth under the other conditions. The results of these experiments are tabulated in Tables 5 and 6.

Curing experiments were carried out also on chlortetracycline resistant transductants of strain 80 and SH5. Phage 80 was propagated on U9 and 4 ml of the transducing phage lysate radiated with ultraviolet radiation having an intensity of 13 ergs/mm^2/sec at a distance of 48 cm. The set of transductants A to G were transduced with the irradiated phage and the set of transductants 1 to 8 obtained with non-irradiated phage. These results are tabulated in Table 7 and 8. Curing of U9 and those transductants produced with irradiated phage was not achieved, but chlortetracycline resistance was lost by strain 2(pen, tet) and by transductants 3 and 6 which were a product of nonirradiated phage.

Kinetics of ultraviolet irradiation of phage lysates

Phage suspension was irradiated for 0, 1, 2, 3, 5 and 7 minutes and then the bacteriophage was used to transduce chlortetracycline, erythromycin, and streptomycin resistance into strain SH5 and 80. The frequency of transduction is

Table 5. Stability of chlortetracycline resistance in strain U9 of Staphylococcus aureus.

Curing conditions	Number colonies examined	Number of sensitive colonies	Colonies losing resistance (%)
Untreated	1539	0	0
Growth at 42.5-43.5 C	1393	0	0
Growth in acriflavine	863	0	0
Growth in ethidium bromide	467	0	0

Table 6. Stability of chlortetracycline resistance in strain 2 (pen, tet) of Staphylococcus aureus.

Curing conditions	Number of colonies examined	Number of sensitive colonies	Colonies losing resistance (%)
Untreated	605	0	0.0
Growth in ethidium bromide	177	2	1.1

Table 7. Stability of chlortetracycline resistance in transductants produced with irradiated phage 80 propagated on Staphylococcus aureus strain U9.

Strain	Curing conditions	Number of colonies examined	Number of sensitive colonies	Colonies losing resistance (%)
1	Untreated	57	0	0.0
1	Growth at 42.5-43.5 C	162	0	0.0
1	Growth in acriflavine	111	0	0.0
2	Untreated	215	0	0.0
2	Growth at 42.5-43.5 C	149	0	0.0
2	Growth in acriflavine	75	0	0.0
3	Untreated	206	0	0.0
3	Growth at 42.5-43.5 C	153	2	1.3
3	Growth in acriflavine	460	0	0.0
4	Untreated	202	0	0.0
4	Growth in acriflavine	494	0	0.0

Table 7. continued.

Strain	Curing conditions	Number of colonies examined	Number of sensitive colonies	Colonies losing resistance (%)
5	Untreated	482	0	0.0
5	Growth at 42.5 43.5 C	44	0	0.0
5	Growth in acriflavine	360	0	0.0
6	Untreated	310	0	0.0
6	Growth at 42.5-43.5 C	33	0	0.0
6	Growth in acriflavine	240	1	0.4
7	Untreated	347	0	0.0
7	Growth in acriflavine	287	0	0.0
8	Untreated	120	0	0.0
8	Growth at 42.5-43.5 C	57	0	0.0
8	Growth in acriflavine	97	0	0.0

Table 8. Stability of chlortetracycline resistance in transductants produced with nonirradiated phage 80 propagated on Staphylococcus aureus strain U9.

Strain	Curing conditions	Number of colonies examined	Number of sensitive colonies	Colonies losing resistance (%)
A	Untreated	105	0	0
A	Growth at 42.5-43.5 C	164	0	0
A	Growth in acriflavine	26	0	0
A	Growth in ethidium bromide	5	0	0
B	Untreated	142	0	0
B	Growth at 42.5-43.5 C	125	0	0
B	Growth in acriflavine	56	0	0
C	Untreated	218	0	0
C	Growth at 42.5-43.5 C	208	0	0
C	Growth in acriflavine	26	0	0
C	Growth in ethidium bromide	19	0	0

Table 8. continued.

Strain	Curing conditions	Number of colonies examined	Number of sensitive colonies	Colonies losing resistance (%)
D	Untreated	195	0	0
D	Growth at 42.5-43.5 C	105	0	0
E	Untreated	177	0	0
E	Growth at 42.5-43.5 C	109	0	0
F	Untreated	220	0	0
F	Growth at 42.5-43.5 C	159	0	0
F	Growth in ethidium bromide	66	0	0
G	Untreated	168	0	0
G	Growth at 42.5-43.5 C	124	0	0
G	Growth in ethidium bromide	130	0	0

plotted against dose of ultraviolet light (Fig. 3 and 4) for strain 80 as recipient strain. When strain SH5 was utilized as the recipient strain similar results were obtained. Small doses of ultraviolet radiation increased the frequency of transduction for resistances to chlortetracycline, erythromycin, and streptomycin with phage 30 when propagated on strain U9.

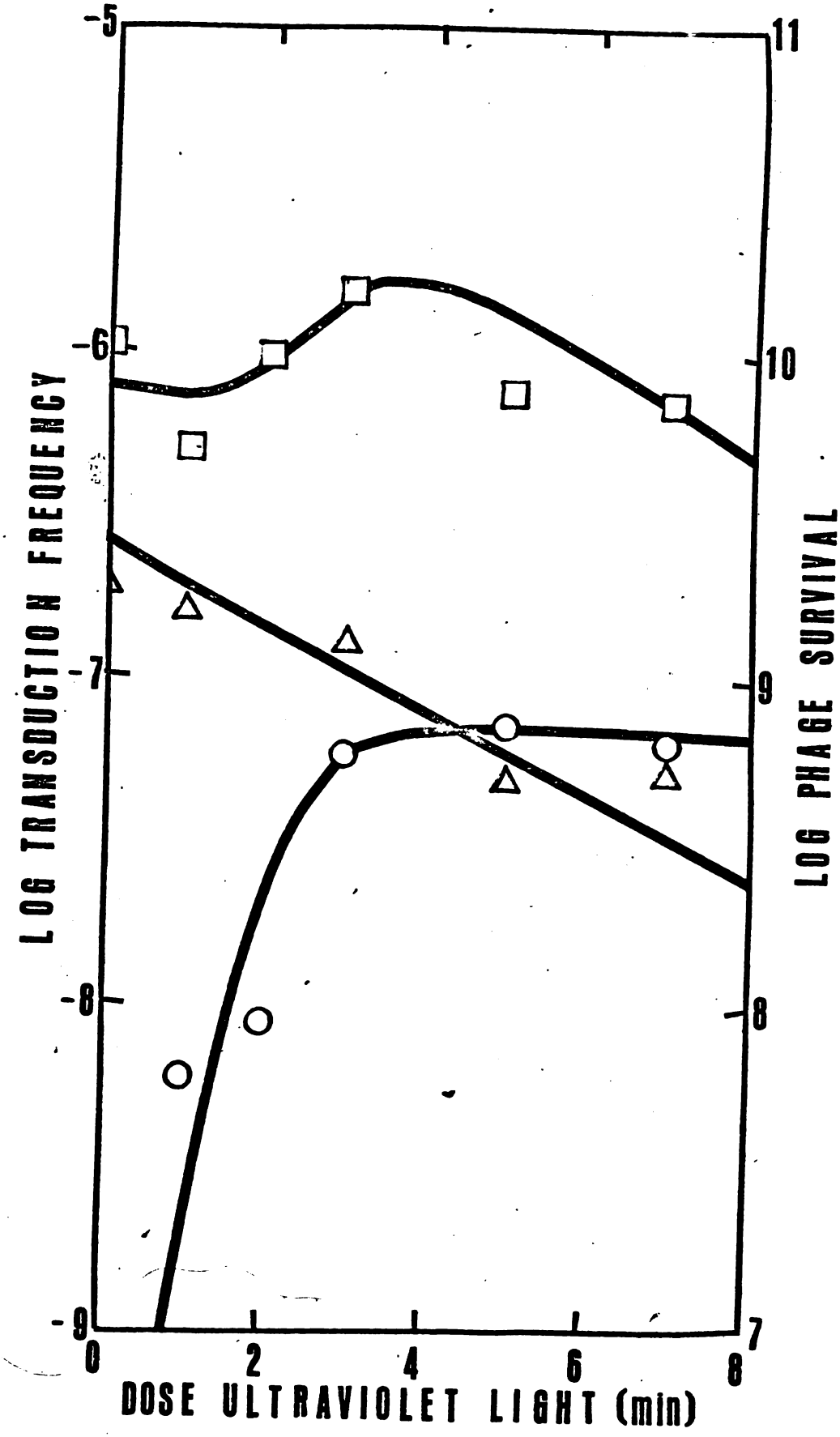
A similar experiment was done utilizing a phage lysate propagated on strain 2(pen, tet) in which the tetracycline marker had been shown by Asheshov (1966-b) to be extrachromosomal. Fig. 5 is the graph of transduction frequency for chlortetracycline resistance versus dosage of ultraviolet radiation of phage lysate propagated on strain 2(pen, tet).

Table 9 is a list of strains utilized in transduction experiments and the transduction frequencies for the different antibiotic resistances which were assayed.

Phenotypic lag

Time required for expression of transduced genes was determined by assaying for erythromycin, streptomycin, and chlortetracycline resistances at hourly intervals after transduction. The number of transductants for each antibiotic resistance is plotted versus time after transduction (Fig. 6). There is a two hour lag before genes

Figure 3. Effect of dose of ultraviolet light on the transduction frequency of chlortetracycline resistance by phage 80 propagated on Staphylococcus aureus strain U9 in which Staphylococcus aureus strain 80 was the recipient. Symbols: (□) transduction frequency of erythromycin resistance, (○) transduction frequency of streptomycin resistance, and (△) phage survival.


-5
-6
-7
-8
-9
11
10
9
8
7
0
2
4
6
8
LOG TRANSDUCTION FREQUENCY
LOG PHAGE SURVIVAL
DOSE ULTRAVIOLET LIGHT (min)

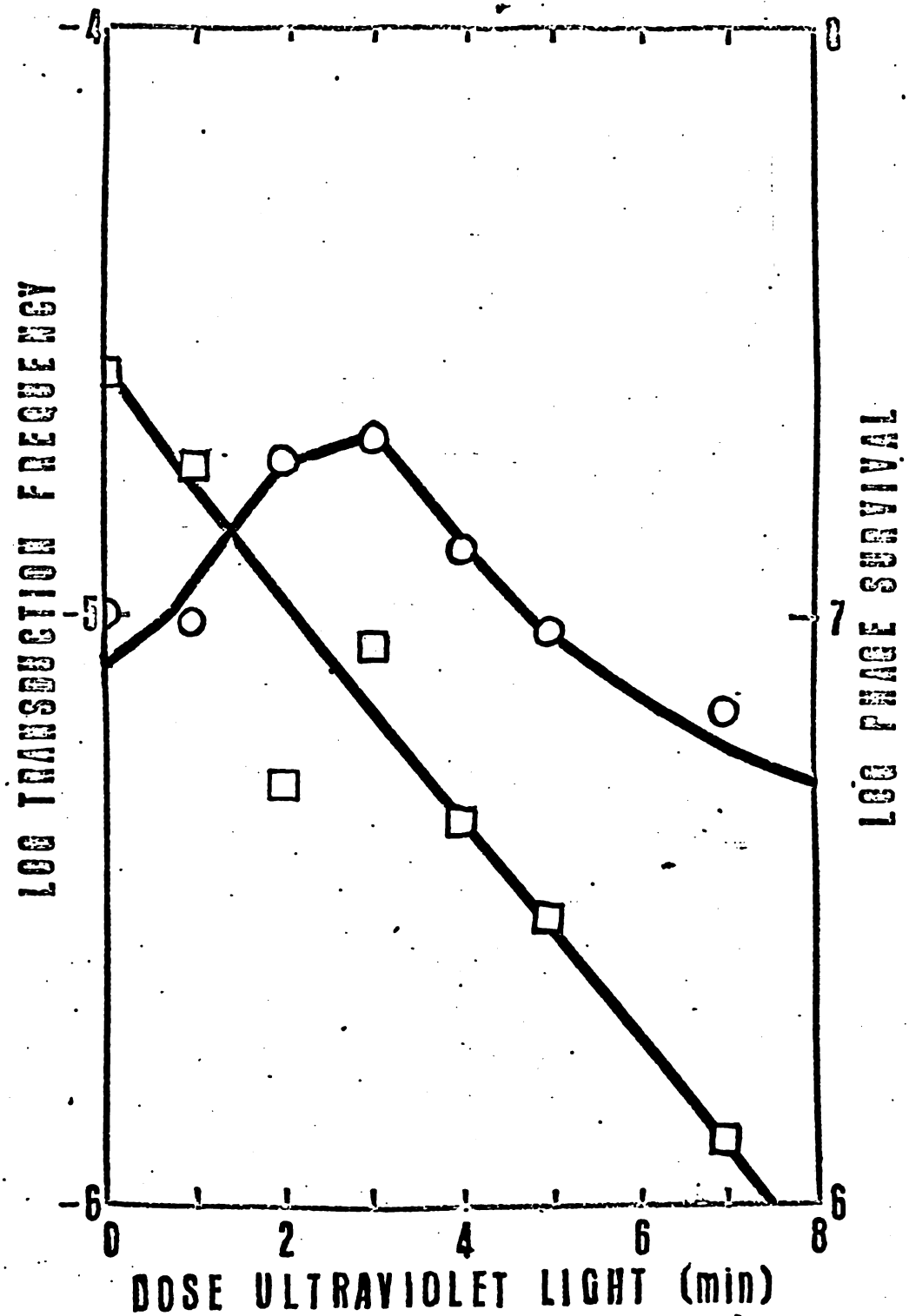


Figure 4. Effect of dose of ultraviolet light on the transduction frequency of chlortetracycline resistance by phage 80 propagated on Staphylococcus aureus strain U9 in which Staphylococcus aureus strain 80 was the recipient. Symbols: (O) transduction frequency of chlortetracycline, (□) phage survival.

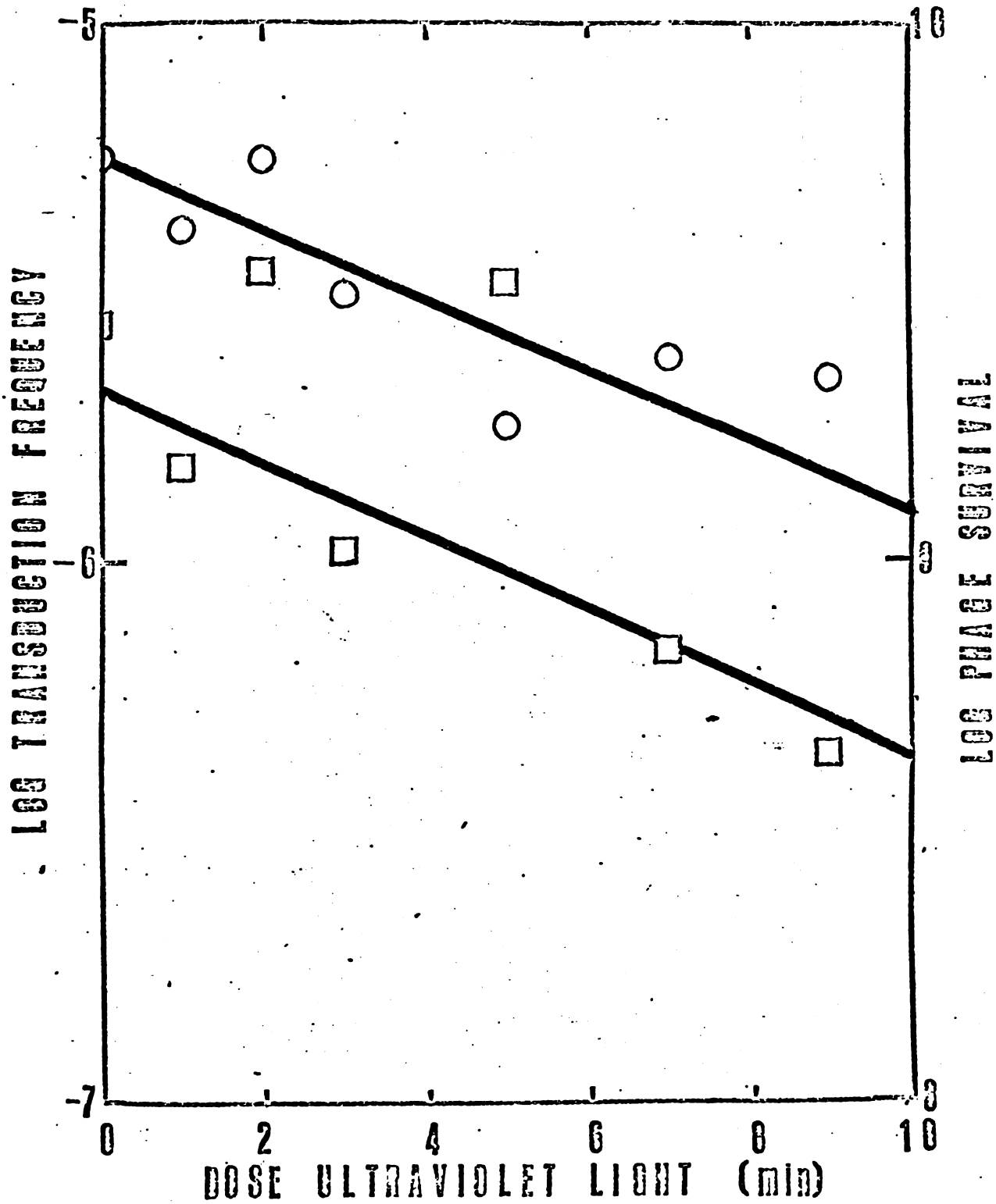
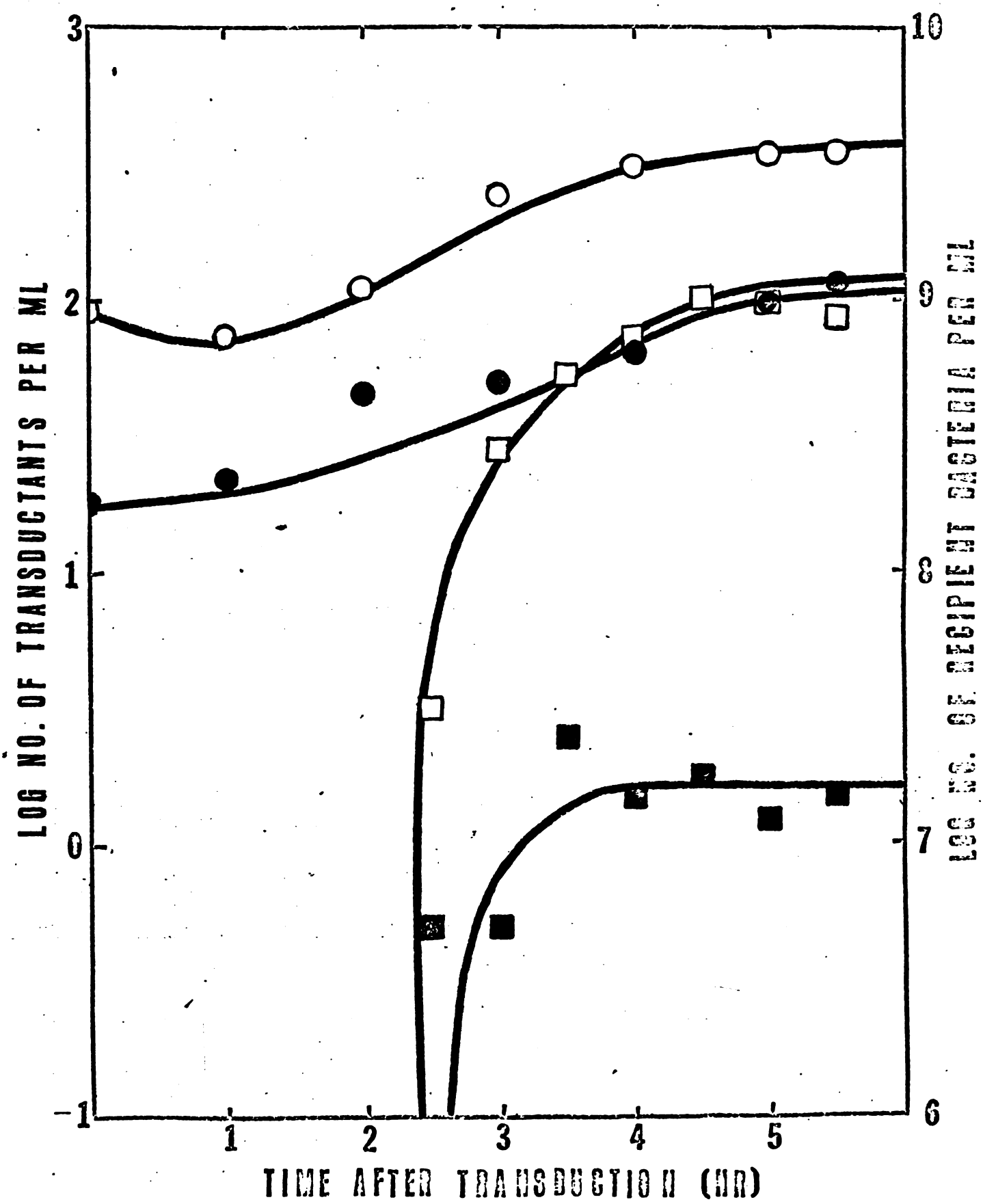


Figure 5. Effect of dose of ultraviolet light on the transduction frequency of chlortetracycline resistance by phage 80 propagated on Staphylococcus aureus strain 2(pen, tet) in which Staphylococcus aureus strain 80 was the recipient. Symbols; (O) transduction frequency of chlortetracycline, (□) phage survival

Table 9. Transduction frequency for antibiotic resistances by phage 80 propagated on Staphylococcus aureus strain U9 and 2(pen, tet) in which Staphylococcus aureus strain 80 was the recipient.

Propagating strain	Transduction of resistance	
	Frequency	Antibiotic
U9	1.0×10^{-6}	Erythromycin
U9	5.8×10^{-9}	Streptomycin
U9	7.9×10^{-6}	Chlortetracycline
U9	9.9×10^{-6}	Chlortetracycline
2	5.6×10^{-6}	Chlortetracycline

Figure 6. Phenotypic lag of transduced resistance to three antibiotics as shown by the time needed for gene expression after transduction. Graphs indicate the number of transductants resistant to chlortetracycline (○), erythromycin (□), and streptomycin (■) determined at hourly intervals after transduction and the number of recipient bacteria per ml (●) as determined by viable cell count at hourly intervals after transduction.


LOG NO. OF TRANSDUCTANTS PER ML
LOG NO. OF RECIPIENT BACTERIA PER ML
TIME AFTER TRANSDUCTION (HR)
3
2
1
0
-1
10
9
8
7
6
1
2
3
4
5

determining erythromycin and streptomycin resistances were expressed. Genes for chlortetracycline resistance in contrast were expressed immediately so that the number of transductants paralleled the viable cell count which was determined hourly.

DISCUSSION

By the use of gradient plate technique bacteria resistant to kanamycin can be selected and isolated but the same method is ineffective for chlortetracycline. On kanamycin gradient plates the number of colonies inversely reflects the gradient of antibiotic concentration. This gradient of growth however does not occur on the chlortetracycline gradient plates and thus strains with increased resistance are not obtained. It appears that the chlortetracycline level of resistance is uniform within the strain and that mutations to tetracycline resistance are exceedingly rare whereas mutations to slight increases in kanamycin resistance do occur and do yield a significant number of resistant variants. Such selected mutations have high probability of being chromosomal mutations as most of the DNA of the bacteria is chromosomal and the chromosome is an essential part of all cells. Plasmids or episomes are not essential for the maintenance of cell viability.

Curing experiments are not successful in eliminating chlortetracycline resistance from strain U9 but do eliminate the resistance in strain 2(pen, tet) which has been shown by Asheshov (1966) to be extrachromosomal. It is concluded that tetracycline resistance is stable in U9 because 4262 colonies were examined and no sensitive colonies were disclosed.

Ultraviolet irradiation of transducing phage lysate increases the transduction frequency for resistance to chlortetracycline, erythromycin, and streptomycin when phage 80 has been propagated on U9. If phage 80 has been propagated on strain 2(pen, tet), irradiation of phage suspension does not increase the transduction frequency of chlortetracycline resistance but instead there is a decrease of the transduction frequency which parallels phage inactivation. Arber (1960) has shown that ultraviolet irradiation of phage lysate produces an increase in transduction frequency for chromosomal markers and a decrease for episome linked traits. It is apparent that the genetic determinant for chlortetracycline resistance in U9 is either linked to the chromosome or to a plasmid containing DNA homologous to the chromosome.

The transduction frequency for chlortetracycline resistance with phage 80 propagated on U9 is approximately a log higher (Fig. 6) than the transduction frequency for resistances to erythromycin and streptomycin. The transduction frequency for chlortetracycline resistance is 1.5 times larger when phage 80 is propagated on U9 rather than on strain 2(pen, tet). Arber (1960) reports that the transduction frequency of extrachromosomal markers is much higher than that of chromosomal markers as the establishment of plasmid DNA within the recipient does not depend on recombination with the host chromosome.

Chlortetracycline resistant transductants produced with phage 80 propagated on U9 were tested by growing under the curing conditions described previously. A set of 8 transductants were a product of nonirradiated phage and a set of 7 of irradiated phage lysate. Sensitive colonies were isolated only from the group transduced with nonirradiated phage. Since ultraviolet irradiation of transducing phage produces an increase in transduction frequency for chromosomal markers and a decrease in transduction frequency for plasmid linked markers, a heterodiploid having chlortetracycline resistance determinants both chromosomally and extrachromosomally would yield mainly plasmid markers with nonirradiated transducing phage and chromosomal markers with irradiated transducing phage. Such heterodiploidy in U9 would account for the high transduction frequency of chlortetracycline resistance, the homology of this genetic marker with the chromosome as noted by the increase in transduction frequency with low doses of ultraviolet irradiation of transducing phage, and the curing of resistance in transductants produced with nonirradiated phage lysate.

A second alternative could be that chlortetracycline resistance is linked to the chromosome in U9 but that in the transductants there is an extraction of the DNA conferring tetracycline resistance by an interaction of the transductant chromosome with a resident plasmid. The resulting transductants would then possess a plasmid linked resistance for chlortetracycline which might be curable. The coelimin-

ation of resistances to tetracycline and penicillin suggest that the interacting plasmid could be a penicillinase plasmid.

A third alternative is that U9 contains a stable plasmid which has extensive homology with the chromosome. This explanation avoids reasons for such homology such as a chromosomal origin of the plasmid or the incorporation of chromosomal material into the plasmid.

The time required for gene expression of resistances to chlortetracycline, erythromycin, and streptomycin were determined and compared with the results described by Poston (1966). The purpose here was to observe any deviation of chromosomal resistance for chlortetracycline and erythromycin with plasmid borne resistances. Dominance of chlortetracycline resistance linked to both chromosome and plasmid is shown by its immediate expression and inheritance in all daughter cells. Erythromycin and streptomycin require 2 hours after transduction for their expression and thus must be recessive traits.

In summary strain U9 has an unusual genetic determinant for chlortetracycline resistance since it displays properties of both extrachromosomal and chromosomal linkage. Evidence for the extrachromosomal locus of tetracycline resistance in U9 is the high transduction frequency when phage is propagated on U9 and the instability of this tetracycline resistance in some transductants. The chromosomal linkage is evidenced by the stability of tetracycline resistance in

U9 and the rise in transduction frequency when transducing phage propagated on U9 is exposed to low doses of ultraviolet light. U9 might therefore be a heterodiploid with respect to tetracycline resistance

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