CHROMOSOMAL DIFFERENCES BETWEEN CULTURES OF STAPHYLOCOCCUS AUREUS

by

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Joseph Jerald Cole III. CHROMOSOMAL DIFFERENCES BETWEEN CULTURES OF STAPHYLOCOCCUS AUREUS. (Under the Direction of Dr. Wendall E. Allen) Department of Biology, East Carolina University, July, 1974.

Genomic maps of two strains of Staphylococcus aureus, 7-8 (animal origin) and 2 (human origin), were prepared by determining the replication sequence of streptomycin, puromycin, chloramphenicol, neomycin, and mitomycin C-resistance factors by the synchronous chromosomal replication technique. Nitrosoguanidine was used for mutagenesis and chromosomal replication synchronized by treatment with phenethyl alcohol.

The order of replication of the genes tested was the same in both strains of S. aureus. Genes replicating at similar times in both strains showed similar distances between genes. The genes for streptomycin and puromycin resistance, replicating near the origin of chromosomal replication in strain 7-8 and near the terminus in strain 2, had different times of replication and distances between genes.

The position of a gene determined by synchronous chromosomal replication was shown not to vary with concentration, pH, or source of the mutagen. The numbers of mutants obtained after the gene in question had replicated were different with different pH of the solution of nitrosoguanidine.

The point of initiation and the direction of chromosomal replication were different for the two strains of S. aureus. Mechanisms were proposed to explain these differences.

CHROMOSOMAL DIFFERENCES BETWEEN CULTURES OF *STAPHYLOCOCCUS AUREUS*

A Thesis

Presented to

the Faculty of the Department of Biology

In Partial Fulfillment

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Master of Arts in Biology

by

Joseph Jerald Cole III

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Dedicated

to

My Mother and Father

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INTRODUCTION

Preparing a chromosomal map of gram-positive Staphylococcus aureus has not been extensively attempted as has genomic mapping of bacteria like Escherichia and Salmonella. Altenbern (1966, 1968, 1969, 1971a, 1971b) reported mapping a total of eleven genes on two strains of S. aureus obtained from humans using the combined methods of marker frequency analysis and synchronous chromosomal mapping.

Marker frequency analysis, originated by Yoshikawa and Sueoka (1963), and synchronous chromosomal mapping, originated by Stonehill and Hutchison (1966), are the only known methods of genomic mapping for asexual bacteria. Chromosomal maps obtained by applying these two techniques are comparable to those produced by the standard conjugation technique for sexually reproducing bacteria. The synchronous chromosomal mapping technique has the advantage over marker frequency analysis for construction of chromosomal maps since it permits the determination of the time during a single chromosomal replication cycle when each gene is duplicated. Technical complexity of synchronous chromosomal mapping, however, does not allow the determination of the sequence of closely spaced genes on the chromosome as is possible with marker frequency analysis.

Altenbern (1973a) found strains of Staphylococcus aureus and cultures of Staphylococcus epidermis, Streptococcus lactis, and Streptococcus muscae he tested began chromosomal replication at the same site on the chromosome, had relatively the same sequence of genes, and replicated chromosomes in the same direction. Genomic mapping of strains of

S. aureus obtained from lower animals has not been reported. S. aureus strains of animal origin are known to possess characteristics different from those of human origin (Meyer, 1967; Cohen, 1972). This would indicate differences in the chromosomal maps of strains of S. aureus from human and bovine origin could exist. Data previously obtained in our laboratory had indicated the possibility that chromosomal replication in the bovine strain proceeded in the opposite direction from that of human strains of S. aureus. A reliable genomic map of the S. aureus chromosome will be attained only after chromosomes from numerous strains have been mapped and the sequence of genes compared. The experiments presented in this paper were performed to compare the sequence of antibiotic-resistance loci in antibiotic-sensitive cultures of a strain of S. aureus obtained from a human (previously mapped by Altenbern) with a strain of S. aureus obtained from a cow.

LITERATURE REVIEW

Nitrosoguanidine-induced Mutations in Microorganisms

Since the initial report of Adelberg, Mandel, and Chein Ching Chen (1965) of the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for Escherichia coli K-12, the chemical has been used extensively to produce mutations in various microorganisms: Tobacco Mosaic Virus (Singer and Fraenkel-Conrat, 1967), Actinomyces olivaceus (Danylenko and Matselyukh, 1970), Micromonospora (Deretta, Betti, and Polsinelli, 1971), Cylindrospermum (Sharmai and Kumar, 1973). Nitrosoguanidine has been used with gram-negative bacteria to produce multiple phenotypic mutants (Skavronskaya and Pokrovskii, 1973) and O-antigen mutants (Uchida, Matsumoto, and Sasaki, 1974) in Salmonella; conjugation-deficient mutants (Roussel, 1971) and auxotropic mutants (Cerdá-Olmedo and Hanawalt, 1968b; Cerdá-Olmedo, Hanawalt, and Guerola, 1968) in E. coli. Auxotrophic mutants of cultures of acid-fast Mycobacterium have also been obtained after treatment with NTG (Konichova-Radochova and Malek, 1969; Koniek and Malek, 1970). Work done with NTG-induced mutations on gram-positive bacteria has included studies of metabolic pathways in Streptococcus (Chuang and Collins, 1971; Luginbuhl and Gooder, 1972) and production of pigment mutants (Altenbern, 1967b), auxotrophic mutants (Altenbern, 1968, 1969, 1971a, 1971b), extracellular nuclease mutants (Omenn and Friedman, 1970), UV-sensitive mutants (Goering and Pattee, 1971), and enterotoxin-A mutants (Friedman and Howard, 1971) in Staphylococcus aureus.

The mode of action of NTG

Cerdá-Olmedo and Hanawalt (1968a) proposed that diazomethane, the decomposition product of NTG, was the principle agent involved in both the lethal and mutagenic effect on DNA at pH 5.5 and higher. He also found the expression of genetic mutation was independent of DNA replication or repair. Craddock (1968), using 0.2 M sodium phosphate buffer at pH 7.5, found that NTG's major reaction with DNA was the methylation of guanine.

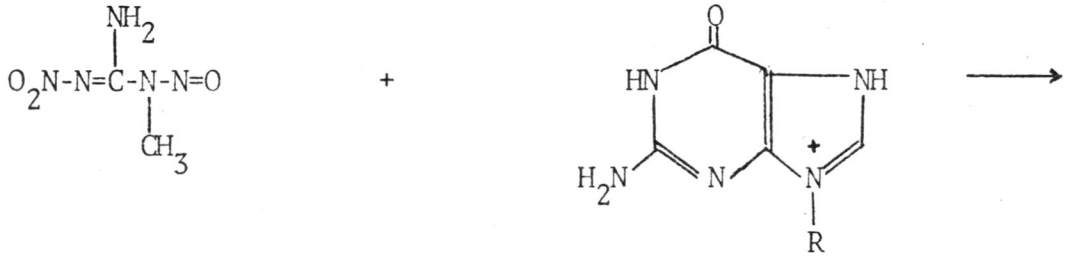
Singer, Fraenkel-Conrat, Greenberg, and Michelson (1968) found that 7-methylguanine was the major product and 1-methyladenine was a minor product of the reaction of NTG on simple polynucleotides in a neutral aqueous solution. No methylation of guanine or adenine was detected in polynucleotides treated with NTG in formamide but some methylated cytosine was found with NTG treatment in dimethyformamide. NTG has its greatest effect on Tobacco Mosaic Virus (TMV) RNA in vitro in a formamide solution and decreasing effects in dimethyformamide and in neutral water, producing large quantities of 3-methyl-cytosine. NTG showed much stronger mutagenic activity on intact TMV particles than on in vitro-treated TMV RNA. By isolating the RNA from viruses treated in vivo they found the guanine and adenine reaction with NTG had been suppressed but that the cytosine reaction had not. From these data, they concluded the methylation of guanine and adenine did not represent the main mutagenic effect of NTG on nucleic acids in live cultures. Singer and Fraenkel-Conrat (1969) working with TMV in vivo also found that reactions observed during treatment of mononucleotides and single-stranded nucleic acids with NTG were not necessarily the same as those reactions observed when double-stranded

nucleic acids and complete viruses were treated with NTG. They suggested that the action of NTG depended on the "base-stacked" conformation of the nucleic acid.

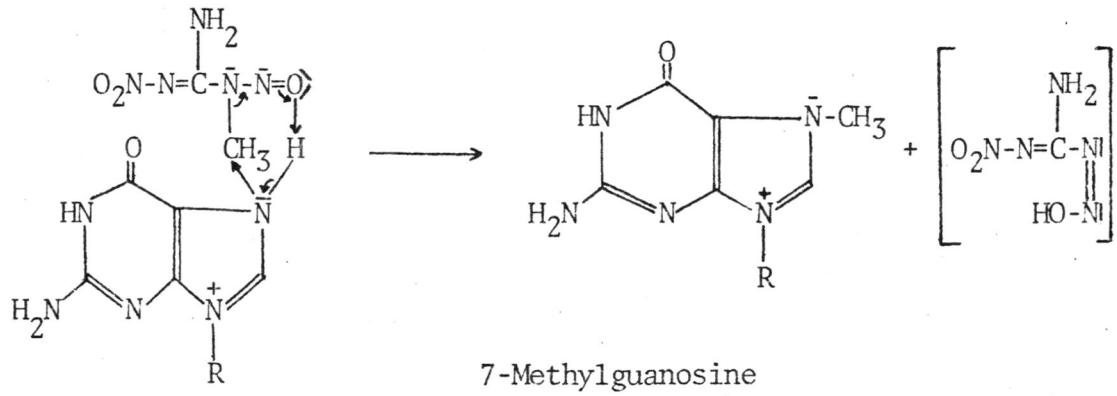
Baker and Tessman (1968) found that the effect of NTG on single-stranded DNA phage S-13 and on double-stranded DNA phage T-4 *in vivo* was different. In single-stranded phage, all four substitution mutations were equally induced by NTG while in double-stranded T-4 phage the GC→AT transition mutations greatly predominated. They concluded these differences in response were due to the molecular environment at the replication point of the DNA; which should depend on the specific mechanism of replication, the nature of the DNA polymerase, and the base composition of the DNA.

Sussmuth, Haerlin, and Lingens (1970, 1972) concluded that the reaction of NTG *in vitro* at pH 6.0 on double-stranded DNA did not involve the formation of diazomethane from NTG as an intermediate, but rather involved the transfer of the methyl group of NTG as an intact unit. These authors used mass spectrometry to determine the mode of action of NTG in mutagenesis of calf thymus DNA *in vitro* and *E. coli* B cells at pH 6.0 *in vivo*. They concluded that the mutagenicity of NTG *in vivo* was due to methylation of certain nucleobases at pH 6.0, caused by a "push-pull" mechanism according to the following reaction.

Methylation of guanine by NTG at pH 5-6 according to Sussmuth et al (1972) (R=DNA)



NTG Guanosine



7-Methylguanosine

These alterations in the nucleic acids would cause a change in the nucleotide sequence of DNA by allowing the mispairing of bases. The presence of specific nucleobases enhanced the mutagenicity of NTG, indicating the compound reacted with specific base sequences in the DNA. The mechanism proposed, however, did not explain a previously determined pH dependence of the methylation reaction observed in vitro (Sussmuth and Lingens, 1968, 1969).

The effect of physical conditions on mutations induced by NTG

As previously described, the specific pH at which NTG is allowed to react with nucleic acids affects the products of the reaction. This has led to experimentation using different buffering solutions, concentrations of NTG, and conditions of exposure to produce mutation in bacteria.

The rate of mutation produced in E. coli, genetically one of the most widely studied species, has been shown to vary with the physical conditions during NTG treatment. Adelberg, Mandel, and Chein Ching Chen (1965) found that the addition of growth media to the mutagenic environment reduced the number of cells surviving the treatment. Llovers and

Cerdá-Olmedo (1973) used treatment with NTG (100 µg/ml) for 30 minutes at 37°C in tris-maleic acid buffer over the pH range of 5.0 to 7.5 and found both survival and mutation rates varied with the pH. The greatest survival occurred at pH 5.5 with a 1,000-fold increase in mutant cells. Each of the following conditions resulted in the production of high survival rates and large numbers of mutants: Adelberg, Mandel, and Chein Ching Chen (1965) used 10, 30, and 100 µg NTG per ml in tris-maleic acid buffer at pH 6.0 for 15 to 30 minutes at 37°C; Cerdá-Olmedo and Hanawalt (1967, 1968a, 1968b) and Cerdá-Olmedo, Hanawalt, and Guerola (1968) used 0.1 mg NTG per ml in tris-maleic acid buffer at pH 5.5 and 1.0 mg NTG per ml in 0.15 M acetate buffer at pH 5.0 for 30 minutes at 25°C; Ward and Glaser (1969a, 1969b) used 0.5 mg NTG per ml in tris-maleic acid buffer at pH 5.5 for 15 minutes at 37°C; and Bostein and Jones (1969) used 100 to 500 µg NTG per ml in 0.1 M citrate buffer at pH 5.5 for 20 minutes at 37°C.

Some authors have used unbuffered solutions of NTG as a mutagenic agent for other species. Altenbern (1973b) produced auxotrophic and antibiotic-resistant mutants in Brucella abortus with 20-minute exposure to NTG (200 µg/ml) in 0.85% saline at 37°C. Jyssum (1969) produced auxotrophic and antibiotic-resistant mutants of Neisseria meningitidis with 20-minute exposure to NTG (3 µg/ml) in 0.85% saline at 37°C. All auxotrophic and antibiotic resistant mutants of Staphylococcus aureus reported by Altenbern (1966, 1967a, 1967b, 1968, 1969, 1971a, 1971b, 1973a) were produced by 20-minute exposure to NTG (200-250 µg/ml) in 0.85% saline at 37°C.

Synchronization of Cell-Division in a Bacterial Population

Phenethyl alcohol inhibition of DNA synthesis

Phenethyl alcohol (PEA), in low concentrations, has been widely used as a means of preventing DNA synthesis in bacterial cultures. Treick and Konetzka (1964) found that 0.32% PEA prevented DNA synthesis in exponential-phase cells of E. coli after a 1.4 to 1.6-fold increase in the amount of DNA present. This increase was thought to represent the completion of the DNA-replication cycle initiated at or prior to the time of PEA addition. Lark and Lark (1966) found that treatment of E. coli 15-T with 0.25% PEA was sufficient to eventually stop DNA synthesis but not RNA or protein synthesis. It was concluded that PEA allowed the completion of the chromosomal replication cycle but prevented initiation of new replication. Altenbern (1966) found that treatment with 0.40% PEA brought about cessation of chromosomal replication in S. aureus after a 1.4 to 1.5-fold increase in total DNA synthesis. Normark (1971) found that 0.05% PEA caused an immediate but transient inhibition of RNA synthesis in E. coli K-12 which lasted approximately ten minutes. The relationship of this effect to the previous report by Lark and Lark (1966) is unclear.

Methods of chromosomal synchronization

Lark (1966) found that thymine starvation, required amino acid starvation, or PEA treatment resulted in the eventual cessation of DNA synthesized by E. coli 15-T cells. Upon release from the inhibition sequential chromosomal replication proceeded from the same region of

the chromosome. Chromosomal replication proceeded at different rates in different growth media and at different temperatures. Lark and Lark (1966) pointed out the concentration of PEA inhibitory for DNA synthesis alone varied with the growth rate and attempts to use PEA to study chromosomal replication should be preceded by studies of the effect of different concentrations of PEA under the specific growing conditions to be used with the strain to be studied. Gutman and Pattee (1970) found that amino acid and thymine starvation of a thymine-dependent auxotroph of S. aureus resulted in synchronous cell growth. Altenbern (1971a) found that PEA treatment, amino acid starvation, or chloramphenicol exposure could be used to cause cessation of DNA replication in S. aureus cells that all began chromosomal replication at apparently the same site upon release from the inhibitory condition.

Cultures of E. coli contained in 0.25% PEA showed inhibition of DNA synthesis but produced large amounts of a protein thought by Lark and Lark (1966) to participate in initiating new cycles of DNA replication. They found this protein alone was not sufficient to initiate the replication cycle and found chloramphenicol (Cml) inhibitory for the protein's synthesis. Lark and Renger (1969) found initiation of a new cycle of DNA synthesis was a three-step process in E. coli. The first step involved the synthesis of a protein inhibited by low concentrations of Cml, but not inhibited by DNA-inhibitory concentrations of PEA. The second step was the synthesis of a protein, inhibited by PEA but not by low concentrations of Cml. The third step in the process was found to involve neither protein nor RNA synthesis. This step was postulated to

involve "a configurational change in the DNA molecule or a change in cell morphology during which the two daughter chromatids are segregated" necessary to initiate DNA replication.

Other effects of PEA

Silver and Wendt (1967) found that 0.25% PEA caused the breakdown of the cellular permeability barrier in E. coli. They concluded that the primary inhibitory effect of PEA on growth was limited to the breakdown of cell membranes. The cell membrane effects were found to be reversible as long as the PEA concentration was below 1.0%. Masker and Eberle (1972) found treatment of E. coli cells with 0.25% PEA did not cause the release of DNA from its membrane association but did prevent DNA replication. Concentrations of 0.5% PEA and greater stopped DNA replication immediately and 25% to 80% of the DNA was released from membrane association.

Treatment with concentrations of PEA slightly above bacteriostatic concentrations (0.45%) resulted in the lysis of actively growing cells of Bacillus subtilis, Escherichia coli, and Staphylococcus aureus (Zyskind and Pattee, 1971). Unexpectedly, cultures of S. aureus showed reduced lysis when treated with concentrations of PEA from 0.8% to 1.0%; 2.0% PEA produced little or no lysis.

Chromosomal Mapping in Bacteria which do not Undergo Sexual Conjugation

At the present time there are two major methods of mapping chromosomes of bacteria which do not undergo sexual conjugation: marker

frequency analysis (MFA) and synchronous chromosomal mapping (SCM). Both methods have been utilized successfully with several species of bacteria.

Marker frequency analysis

The method of marker frequency analysis was first devised by Yoshikawa and Sueoka (1963). Sueoka and Yoshikawa (1965) used the technique for mapping the location of genes on the chromosome of Bacillus subtilis through donor-recipient transformations. Synchronized cells were obtained by starvation of an amino acid dependent mutant. Mutations were obtained by ultraviolet-penicillin treatment. A map of the chromosome was constructed based on the ratios of the numbers of transformants of a gene obtained with DNA from synchronized and non-synchronized (exponential phase) cells.

Altenbern (1971a, 1971b) modified marker frequency analysis. He determined the ratios of the numbers of chemically induced mutations of a gene, rather than transformants, to produce maps of the chromosome of S. aureus. Reference cells with completed chromosomes were obtained with PEA treatment, chloramphenicol treatment, or amino acid starvation. Nitrosoguanidine (NTG) was used to produce mutations. Altenbern (1973a) recently applied marker frequency analysis using NTG mutagenesis for mapping the chromosomes of additional strains and bacterial species. He constructed maps of antibiotic-resistance loci and fermentation loci for S. aureus, S. epidermis, Streptococcus muscae, and Streptococcus lactis. He found no case where the order of genes was totally inverted

(indicating opposite polarities of replication) compared to the sequence in other strains of the same species or in another species. Altenbern (1973b) also produced a partial genomic map of Brucella abortus by this method.

Synchronous chromosomal mapping

Stonehill and Hutchison (1966) originated the method of synchronous chromosomal mapping and used it to prepare a chromosomal map of Streptococcus faecalis. Cells were synchronized by amino acid starvation and mutants were induced by UV radiation. A map showing the loci of genes for antibiotic and bacteriophage resistance was constructed.

Synchronous chromosomal mapping was used by Cerdá-Olmedo and Hanawalt (1968b) to prepare a chromosomal map for E. coli. The cells were synchronized by 90 minutes of amino acid starvation followed by 50 minutes of thymine starvation. Sequential mutagenesis of the synchronized cells during one growth cycle was performed by periodic exposure of samples to NTG (0.1 mg/ml) for 30 minutes at 25°C in tris-maleic acid buffer at pH 5.5. Maxima of mutations for different markers were observed to occur at a particular time in the replication cycle indicating the relative position of genes. The map produced conformed to maps of the chromosome of E. coli composed by using more conventional methods. Ward and Glaser (1969a, 1969b) used SCM to produce a chromosomal map of E. coli. They used a membrane collection technique and amino acid starvation to synchronize the cells. NTG was used as the mutagen. They found the determination of the back-mutation rate of a given auxotrophic marker as a function of the cell's age could be used

to determine the time at which the gene was being replicated. Working with E. coli, Bostein and Jones (1969) found stationary-phase cells initiated DNA replication at a unique point on the chromosome, replication proceeded in one direction, and NTG action was on the replication point. They also found NTG mutants derived from stationary cultures carried mutations mapping only in the left half of the chromosomal map while mutagenesis of exponentially growing cultures produced mutation of genes distributed over the entire chromosome. Cerdá-Olmedo, Hanawalt, and Guerola (1968) also used synchronous chromosomal mapping to produce a map of the E. coli chromosome. The cells were synchronized by thymine starvation and mutation was produced with NTG. All the genes mapped were amino acid requirements except for streptomycin resistance.

Jyssum (1969) used the synchronous chromosomal mapping technique to map the chromosome of Neisseria meningitidis. He induced mutants by NTG treatment during synchronous replication after release from prolonged chloramphenicol inhibition. The replication map he produced by this method agreed with maps produced by other methods. The mutation resulting in the change from genetic competence to genetic incompetence, however, resulted in a change in replication origin as well as in direction of chromosomal replication in this species.

Chromosomal mapping of Staphylococcus aureus

There is a paucity of literature reporting transformation of S. aureus. (Imshemstiskii and Perova, 1959; Lambina, 1961; Komarov, 1962; Smirnova, Gubendo, and Ribankowa, 1969; Lindberg, Sjostrom and Johansson, 1972; Lindberg and Novic, 1973; Rudin, Sjostrom, Lindberg, and Philipson,

1974). Chromosomal mapping using transformation has not been reported due to difficulties involved in the process.

Altenbern (1966, 1967a, 1968, 1969, 1971a) has applied the technique of synchronous chromosomal mapping to S. aureus. Synchronous DNA replication was produced by treatment with 0.40% PEA, and mutations were induced by treatment with NTG (200 µg/ml) at 10 to 20-minute intervals. He also found a reduction in incubation temperature from 37°C to 30°C during the synchronized growth cycle was necessary to increase the generation times of the bacteria to accommodate the technical manipulations of the mapping procedure (Altenbern, 1966). The time required for total genomic replication under these conditions was 60 minutes. Genes mapped included guanine, riboflavin, pigmentation, tryptophane, pantothenate, and resistance to nitrosofurazone, novobiocin, and chloramphenicol.

Altenbern (1968) modified the above procedure to provide a 120-minute replication time. The chromosomal map included all the above-mentioned markers except pigmentation (Altenbern, 1966) and included, additionally, the locus for vancomycin resistance. Altenbern (1969) presented replication maps for 6 strains of S. aureus constructed by SCM. He reported three of these strains contained a multiple-resistance factor (MRF). The MRF-containing strains took longer to replicate than those strains not containing MRF. No new loci were reported.

Only Altenbern has presented genomic maps using synchronous chromosomal mapping and marker frequency analysis for the same strains. Altenbern (1967a) constructed a partial genomic map for S. aureus

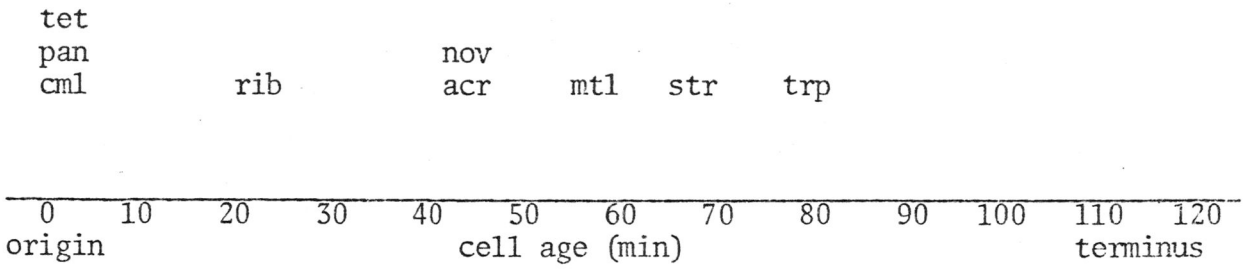
strain "Maybush" using the SCM and MFA techniques. To account for the apparent shifting of the loci of genes during MFA, he proposed strain "Maybush" and probably other strains of S. aureus contained two replicons, different numbers of copies of each being present in different phases of growth. The loci of the genes for pantothenate, acriflavin, and mutation 21 occurred in one replicon while the loci of the genes for guanine, chloramphenicol resistance, riboflavin, novobiocin resistance, nitrofurazone resistance, pigmentation, and tryptophan occurred in the other.

Altenbern (1971a), using MFA, found the two replicons in S. aureus could not replicate independently of each other, and more than one replication point occurred in chromosomes of cells during logarithmic growth in non-synthetic medium. Altenbern (1971a) reported the order of 5 of the above genes obtained by MFA agreed with their position determined by synchronous chromosomal mapping.

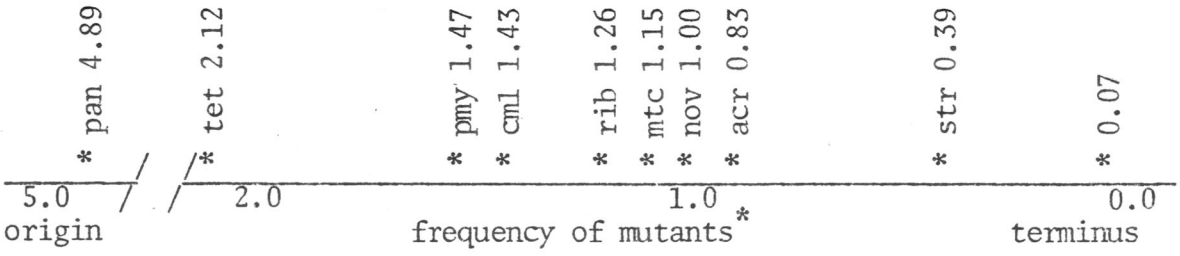
Altenbern (1971b) presented an expanded chromosomal map of loci for drug resistance in two strains of S. aureus. He obtained excellent agreement in the positions of genes obtained by SCM and MFA. He found the order of genes derived by either method were the same. The chromosomal map presented by Altenbern (1971b) for S. aureus strain 2 is presented on the following page.

Proposed chromosomal map of S. aureus strain 2
(Robert A. Altenbern, 1971b)

By synchronous chromosomal mapping



By marker frequency analysis



(*Obtained from comparison of numbers of mutant colonies for each locus in synchronized and non-synchronized cells normalized to a ratio of 1.43 for comparison of Cml mutants to Nov mutants.)

MATERIALS AND METHODS

Cultures

Staphylococcus aureus strain 7-8 was isolated from the teat of a cow with mastitis. Staphylococcus aureus strain 2 was kindly provided by Robert A. Altenbern, Pathology Division, U.S. Army Medican Research Institute of Infectious Diseases, Fort Detrick, Fredrick, Maryland.

The phage-sensitivity pattern for strain 7-8 is 42E/52A/53/75/77/79 and for strain 2 is 42E/54/75/81. Bacteriophage propagation and typing was according to the methods of Blair and Williams (1961). The twenty bacteriophages used were all of the International Phage Typing Series obtained from the Sylvana Company, Milburn, New Jersey.

Media

Trypticase Soy Agar, Baltimore Biological Laboratories (BBL), Baltimore, Maryland, containing 0.5% (w/v) Yeast Extract (BBL) was the basal medium used in these experiments. This medium was referred to as TSAY. Trypticase Soy Broth (TSB, BBL) was the liquid medium used. Yeast Extract (0.5%, w/v) was added to some of the TSB. This medium was designated TSBY.

Stock cultures were maintained on TSAY slants stored at $4^{o}C$. Transfers to fresh media were made at 3-month intervals.

Chemicals and Reagents

Phenethyl alcohol (PEA) was purchased from Eastman Kodak Company, Rochester, New York. It was sterilized by passage through a 0.45 μ filter (Falcon Plastics, Los Angeles, Calif.) and stored at 4°C in an amber bottle until used.

Samples of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, and Sigma Chemical Company, St. Louis, Missouri. The NTG was stored desiccated at -5°C until use. Solutions of NTG were made daily in either 0.85% (w/v) NaCl solutions or 1 M potassium-phthalate ($2\text{-HOCOC}_6\text{H}_4\text{COOK}$) buffer solution titrated to pH 5.5 with 1 N NaOH and sterilized by filtration as above. The solutions were stored in the dark at 4°C until used.

Antibiotics used in the experiment include Streptomycin Sulphate (Str), Eli Lilly Company, Indianapolis, Ill.; Mitomycin C (Mtc), Sigma Chemical Company; Chloramphenicol (Cml), Sigma Chemical Company; Puromycin Dihydrochloride (Pym), Sigma Chemical Company; and Neomycin Sulfate (Neo), Sigma Chemical Company. All antibiotics were prepared in useable concentrations by dilution with sterile saline (0.85%, w/v, NaCl) and stored at -5°C. Final dilution was prepared in melted and cooled (50°C) TSAY. Plates were then poured and used within 24 hours.

Burton's Diphenylamine Reagent (Burton, 1956) was prepared by dissolving 1.5 gm of steam-distilled diphenylamine (Fisher Scientific Co., Indicator Grade) in 100 ml of redistilled acetic acid (Fisher, Reagent) and 1.5 ml of concentrated sulfuric acid (Fisher, Reagent).

The solution was stored in the dark. At the time of use, 0.10 ml of aqueous acetaldehyde (Eastman Kodak Co., Reagent, 16 mg/ml) was added for each 20 ml of reagent required.

DNA Standard Curve

A stock solution was prepared containing 200 µg of DNA (Calf thymus Type V, Sigma Chemical Company) per ml distilled water. Dilutions containing 150, 100, and 50 µg of DNA per ml distilled water were prepared from the stock. Colorimetric determination of the DNA concentration was performed by mixing 1 ml of each DNA standard with 2 ml of Burton's reagent in a spectrophotometer tube. The control consisted of a tube containing 1 ml of water and 2 ml of the reagent. All tubes were incubated for exactly 18 hours at 30°C for color development. The absorbance (600 nm) of each mixture was determined spectrophotometrically (Bausch and Lomb, Spectronic 20). The results of duplicate tests were averaged and the average absorbance (abscissa) plotted against the concentration of DNA (ordinate).

PEA-induced Cessation of DNA Synthesis

One loopful of growth from overnight cultures of strains 7-8 and 2 on TSAY was transferred into separate tubes containing 10 ml of TSB and incubated at 37°C for 10 to 14 hours. The contents of each tube were then added to flasks containing 100 ml of fresh TSBY and incubated for 4 to 6 hours with shaking (90 cycles/min) in a water bath at 37°C (Forma Scientific, Model 2562). A volume of 50 ml was withdrawn from the cultures to use as a control and 0.22 ml of PEA (0.40%, v/v) was

added to the remainder. Each culture was shaken vigorously by hand for 6 minutes. This is time "B". Turbidities of all cultures were equilibrated colorimetrically (Klett-Summerson, Model 800-3, 540 nm) and the cultures were maintained at 30°C for the following 150-minute period. At time "B" and each 30 minutes thereafter for the next 150 minutes, 5-ml samples were removed from each PEA-treated and untreated culture, and centrifuged (Sorval RC-2, 5°C) at 12,000 x g for 10 minutes. The supernatant fluids were discarded and cells were washed twice with 5-ml portions of cold 5% (v/v) trichloroacetic acid (TCA) at 4°C.

The DNA was extracted from the cells with 2.5 ml of 5% TCA at 90°C for 15 minutes. Resulting suspensions were centrifuged at 1,500 x g for 10 minutes and 2 ml of each supernatant fluid decanted into colorimetric tubes. Into these tubes of extracted DNA and a control tube containing 2.0 ml of 5% TCA was added 0.5 ml of 25 N $HClO_4$ and two volumes (5 ml) of Burton's reagent. The tubes were incubated at 30°C for exactly 18 hours. After the incubation period, the absorbance (600 nm) of each sample was determined (Beckman, Model D B Spectrophotometer). The DNA concentration of these extracts from duplicate experiments was determined from the standard curve for DNA prepared before.

The Effect of NTG on S. aureus Strains 7-8 and 2

Cultures of S. aureus strains 7-8 and 2 were grown overnight in 10 ml of TSB at 37°C. The turbidity of the cultures was equilibrated colorimetrically (Klett-Summerson, Model 800-3, 540 nm). Duplicate 3-ml samples from each culture were transferred into separate test tubes. A 1-ml volume of a saline solution of NTG (800 µg/ml) from

Sigma Chemical Company was added to 1 tube of each culture and the same amount of NTG from Aldrich Chemical Company was added to 1 tube of each culture. The mixtures were incubated at 30°C for 20 minutes followed by the addition of 10 ml of saline. The diluted suspensions were centrifuged (1,500 x g, 20 minutes), the supernatant fluids discarded, and the cells resuspended in 1/2 strength TSBY. Following overnight incubation at 37°C in 5% CO_2-95% air, samples were diluted and 0.1 ml amounts spread over surfaces of TSAY plates. Controls consisted of a 3-ml sample of each strain added to 1 ml saline and treated as above. The plates were incubated at 37°C in 5% CO_2-95% air for 24-48 hours, and the numbers of colonies resulting plotted against the concentration of NTG.

In other experiments, samples of the two strains were each exposed to 0, 100, 200, and 800 µg of NTG per ml from Sigma Chemical Co. and Aldrich Chemical Co. and treated as above except the samples were spread on duplicate plates of TSAY containing 4 µg/ml Cml. All plates were incubated and colony counts determined as before.

Chromosomal Mapping by Sequential Chemical Mutagenesis of a Synchronized Population of S. aureus

Cultures of strains 7-8 and 2 were grown and treated with PEA as previously described. Following the addition of PEA (time "B"), the PEA-treated cultures and controls were incubated at 30°C for 2 hours without shaking to allow for the completion of DNA replication in the PEA-treated cultures. Following the 120-minute incubation period, the

cells were centrifuged (Sorval RC-2, 5°C) at 12,000 x g for 15 minutes then resuspended in 100 ml of 1/2 strength TSBY and placed at 30°C. This is time "0".

The effect of amount of NTG on the numbers of Cml and Mtc-resistant mutants produced and the position of these loci on the chromosome determined by sequential chromosomal mapping was determined as follows. At time "0" and every 20 minutes thereafter for the following 120 minutes, 3 ml of each culture (cells with PEA treatment and controls) was placed in a sterile 15-ml centrifuge tube containing 1.0 ml of saline, 1.0 ml of NTG (800 µg/ml) in saline (final concentration of NTG, 200 µg/ml), or 1.0 ml of NTG (3,200 µg/ml) in saline (final concentration of NTG, 800 µg/ml).

The effect of buffered and non-buffered solutions of NTG on the numbers of Cml-resistant mutants produced and position of the cml locus was determined by a modification of this procedure. At "0" time and every 20 minutes thereafter for the 120-minute period, the 3-ml samples were centrifuged (1,500 x g, 15 minutes), the supernatant fluid discarded, and the cells resuspended in 4-ml of NTG (200 µg/ml) dissolved in saline or dissolved in 1 M potassium-phthalate buffer (pH 5.5).

All suspensions from the above procedures were then incubated at 30°C for 20 minutes, followed by the addition of 10 ml of cold saline. The suspensions were centrifuged at 1,500 x g for 20 minutes at room temperature, the supernatant fluids discarded, and the sediments stored at 5°C until all samples had been collected. To each tube was added 1 ml of fresh TSBY. The tubes were shaken gently to resuspend the

cells and incubated overnight (12-15 hours) in 5% CO_2-95% air at 37°C. Dilutions were prepared in sterile saline and plated in duplicate on TSAY plates containing either 4 µg/ml Cml of 1 µg/ml Mtc.

Subsequent experiments included the following modifications of the above procedure. Prior to the addition of NTG, the cells were centrifuged at 1,500 x g for 10 minutes at room temperature, the supernatant fluid discarded, and the cells resuspended in 4 ml of 1M potassium-phthalate buffer (pH 5.5) containing 200 or 800 µg/ml NTG. The samples withdrawn contained 2 ml of cell suspension and were treated as before. Samples were taken at either 10 or 20-minute intervals. The TSAY plates contained either Cml, 4 µg/ml; Pym, 10 µg/ml; Mtc, 1 µg/ml; Str, 10 µg/ml; or Neo, 1.5 µg/ml.

All plates were incubated at 37°C in 5% CO_2-95% air until colonies developed. The average number of colonies occurring on duplicate plates of the same dilution was plotted against the time of exposure to NTG after release from PEA inhibition. The time at which the number of a particular type of mutant first increased was considered to be the time of replication of the gene in question and indicated the distance, in time units, of the gene from the point of origin of the chromosome. Each gene locus was determined a minimum of three times and the values presented in "Results" represent an average of data from three separate experiments.

RESULTS

Figure 1 shows the effect of 0.40% phenethyl alcohol (PEA) on DNA synthesis by Staphylococcus aureus strains 7-8 and 2. The control cultures of both strains containing to PEA produced over twice the amount of DNA as did the treated cultures for each strain respectively during the 180-minute period of testing. PEA had effectively inhibited DNA synthesis within 90 minutes after its addition to cultures of both strains 7-8 and 2.

Plots of the numbers of Cml-resistant mutants induced by periodic treatment of non-synchronized (no PEA treatment) cultures of strains 7-8 and 2 with NTG revealed the numbers of mutants recovered throughout the 120-minute testing period remained constant for each concentration of NTG (Figure 2, curves 1 and 5). However, the number of mutants recovered after exposure to high concentrations (800 µg/ml) of NTG was almost 2,500-fold less than the number recovered with exposure to NTG at 200 µg/ml (Figure 2, curves 1 and 5). Distinct points of increase in numbers of Cml mutants of synchronized (PEA treated) cultures occurred at 30-40 minutes (Figure 2, curves 2 and 6) and 120-0 minutes (Figure 2, curves 3 and 4) respectively for strains 7-8 and 2, indicating the points of replication of the cml genes. The point of replication of the gene for cml of either culture did not vary with the concentration of NTG.

Figure 3a shows the effect of different samples of NTG (Sigma Chemical Co. and Aldrich Chemical Co.) on the viable-cell count of cultures of S. aureus strains 7-8 and 2. Strain 7-8 had 1-log less

Figure 1. Phenethyl alcohol-induced inhibition of DNA synthesis in S. aureus strains 7-8 and 2

Legend:

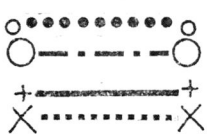


Figure 2. Comparison of the numbers of chloramphenicol-resistant mutants of S. aureus strains 7-8 and 2 obtained upon sequential treatment of synchronized and non-synchronized cultures with nitrosoguanidine (NTG) at a concentration of 200 and 800 µg/ml

Legend:

(1) Strains 7-8 and 2 treated with NTG (200 µg/ml) not treated with PEA

(2) Strain 7-8 treated with NTG (200 µg/ml) and synchronized with PEA

(3) Strain 2 treated with NTG (200 µg/ml) and synchronized with PEA

(4) Strain 2 treated with NTG (800 µg/ml) and synchronized with PEA

(5) Strains 7-8 and 2 treated with NTG (800 µg/ml) not treated with PEA

(6) Strain 7-8 treated with NTG (800 µg/ml) and synchronized with PEA

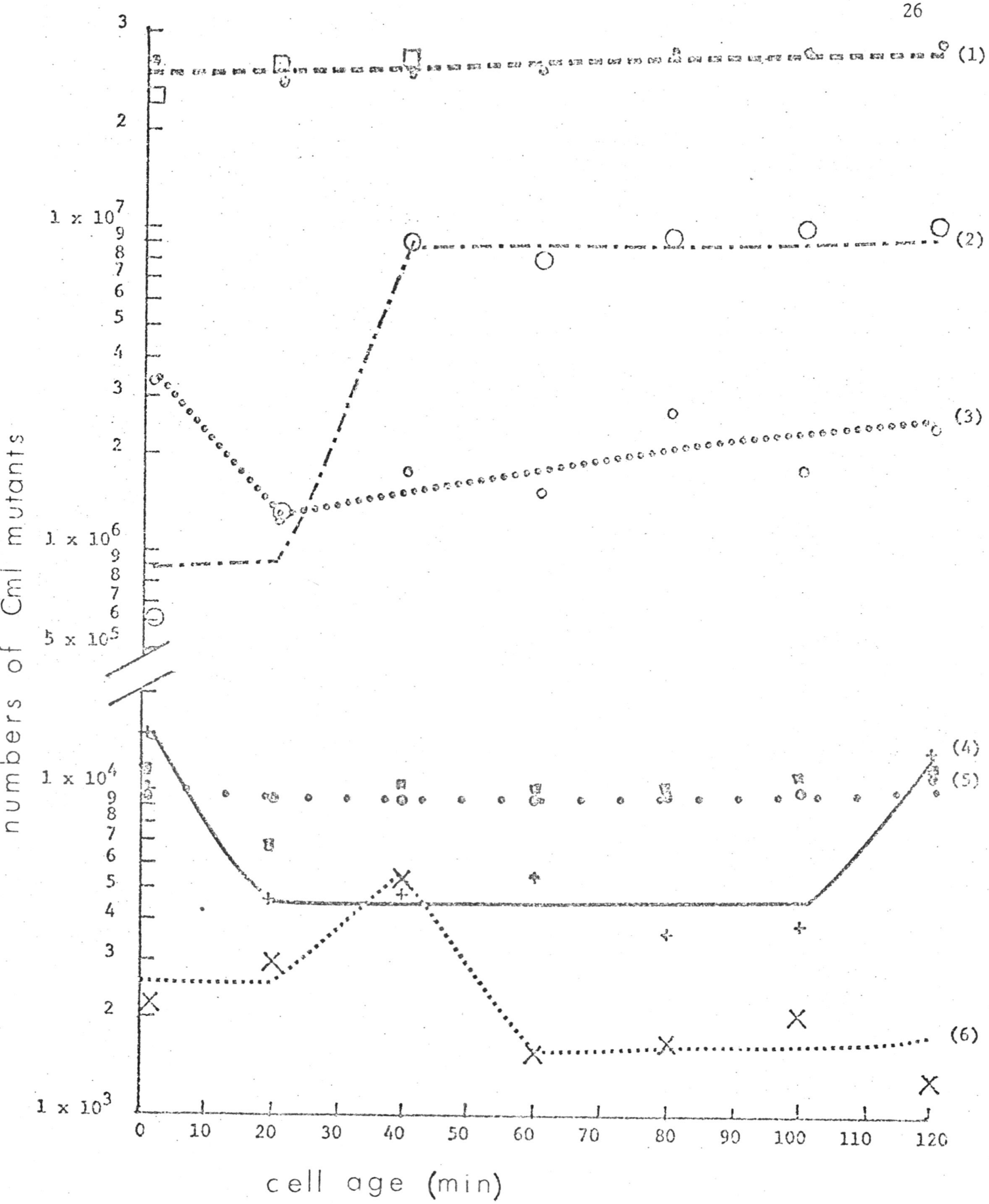
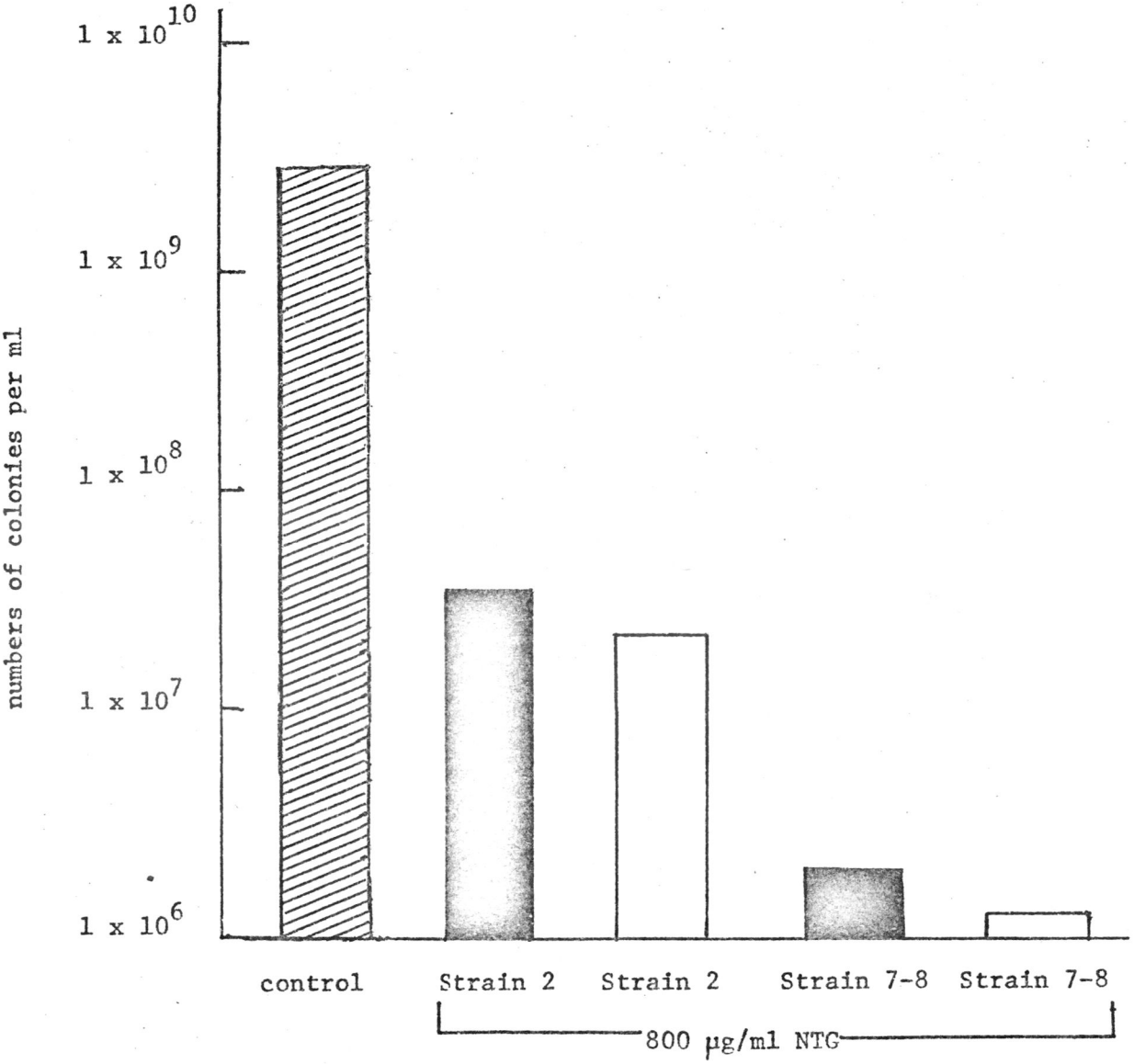

numbers of Cml mutants
cell age (min)
(1)
(2)
(3)
(4)
(5)
(6)
3
2
1 x 10^7
9
8
7
6
5
4
3
2
1 x 10^6
9
8
7
6
5 x 10^5
1 x 10^4
9
8
7
6
5
4
3
2
1 x 10^3
0
10
20
30
40
50
60
70
80
90
100
110
120

Figure 3a. The effect of nitrosoguanidine on viability of S. aureus strains 7-8 and 2



Legend:

Control indicates strains 7-8 and 2 not treated with NTG
Black indicates NTG from Aldrich Chemical Co.
White indicates NTG from Sigma Chemical Co.

survivors than strain 2 following 20-minute exposure to NTG (800 µg/ml). Treatment with NTG from Sigma Chemical Company killed twice as many cells as did treatment with NTG from Aldrich Chemical Company.

Figure 3b shows the numbers of Cml mutants obtained from non-synchronized cultures of the two bacterial strains after exposure to three concentrations of the two different preparations of NTG for 20 minutes. The initial numbers of viable cells were equal for both cultures. The numbers of Cml mutants obtained from the stationary phase cultures of strain 7-8 and 2 varied with the concentration of NTG used for mutagenesis. There was an average 10-fold difference in numbers of Cml mutants comparing strain 7-8 with strain 2 upon exposure to 100 µg NTG per ml. An average of 50 times as many Cml mutants were obtained from strain 7-8 as from strain 2 by exposure to 200 µg NTG per ml. An average of 4 times as many Cml mutants were obtained from strain 7-8 as from strain 2 with 800 µg NTG per ml. The greater lethal effect of the NTG obtained from Sigma Chemical Company reported above was verified.

Saline solutions of equal concentrations of NTG (200 µg/ml) obtained from Aldrich Chemical Company and Sigma Chemical Company had a pH of 6.9 and 7.65 respectively. Cells of strains 7-8 and 2 exposed to 200 µg/ml NTG at pH 5.5 showed both high survival rates (70% in buffer at pH 5.5 as compared to 41% in saline) and high mutation rates for Cml resistance (Figure 4). A comparison of the location of the cml locus obtained for S. aureus strains 7-8 and 2 using 200 µg/ml NTG dissolved in saline and in potassium-phthalate buffer at pH 5.5 is

Figure 3b. The effect of NTG on numbers of Cml-resistant mutants of S. aureus strains 7-8 and 2

Legend:

⊗ Initial cell titer per ml (plated on TSAY)
(1) Strain 7-8 treated with NTG obtained from Aldrich Chemical Co.
(2) Strain 7-8 treated with NTG obtained from Sigma Chemical Co.
(3) Strain 2 treated with NTG obtained from Aldrich Chemical Co.
(4) Strain 2 treated with NTG obtained from Sigma Chemical Co.

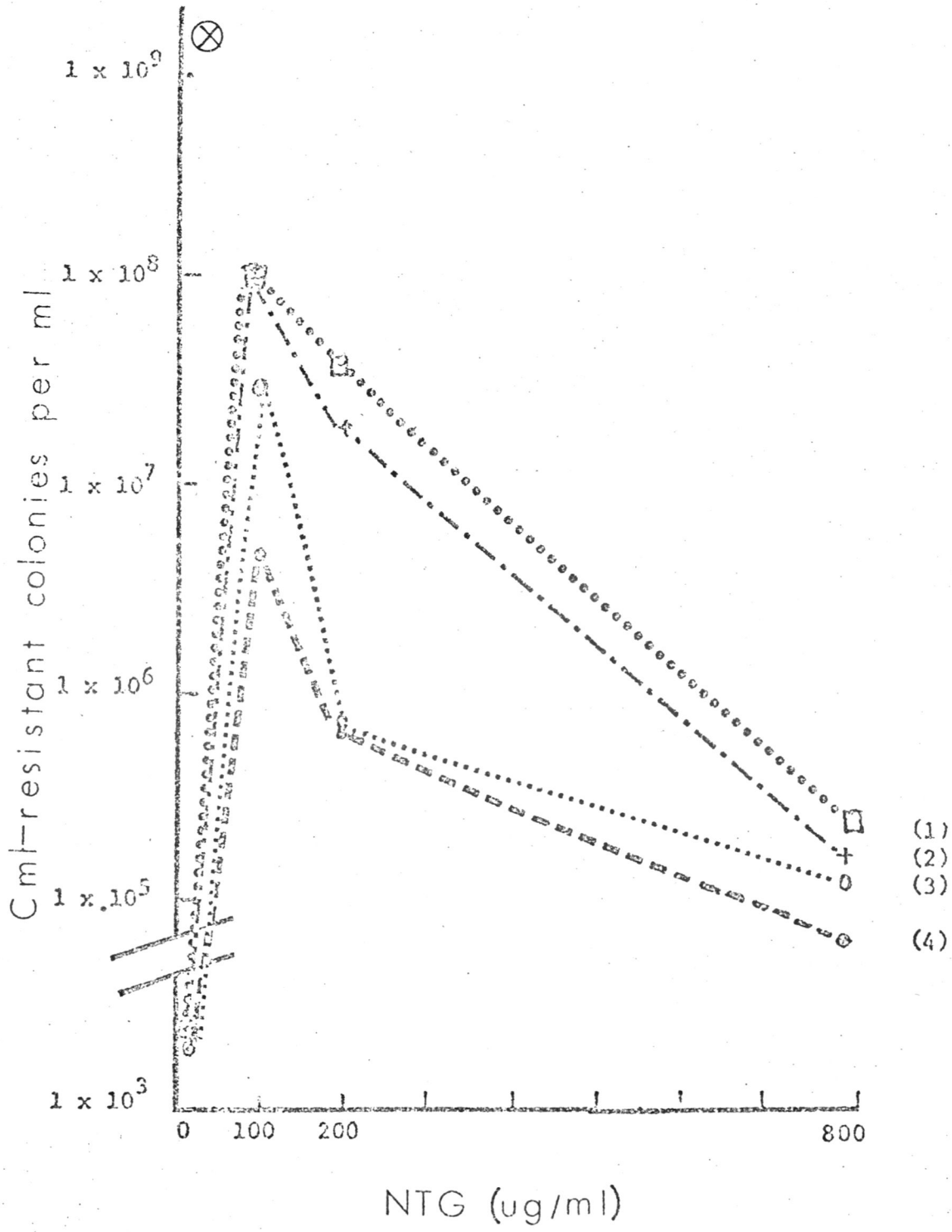
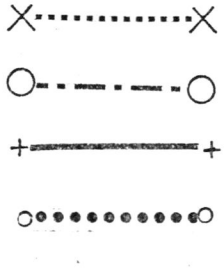

1 x 10^9
1 x 10^8
1 x 10^7
1 x 10^6
1 x 10^5
1 x 10^3
Cm-resistant colonies per ml
0
100
200
800
NTG (ug/ml)
(1)
(2)
(3)
(4)

Figure 4. Effect of buffering on the frequency of Cml-resistant mutants of S. aureus strains 7-8 and 2 induced by NTG

Legend:

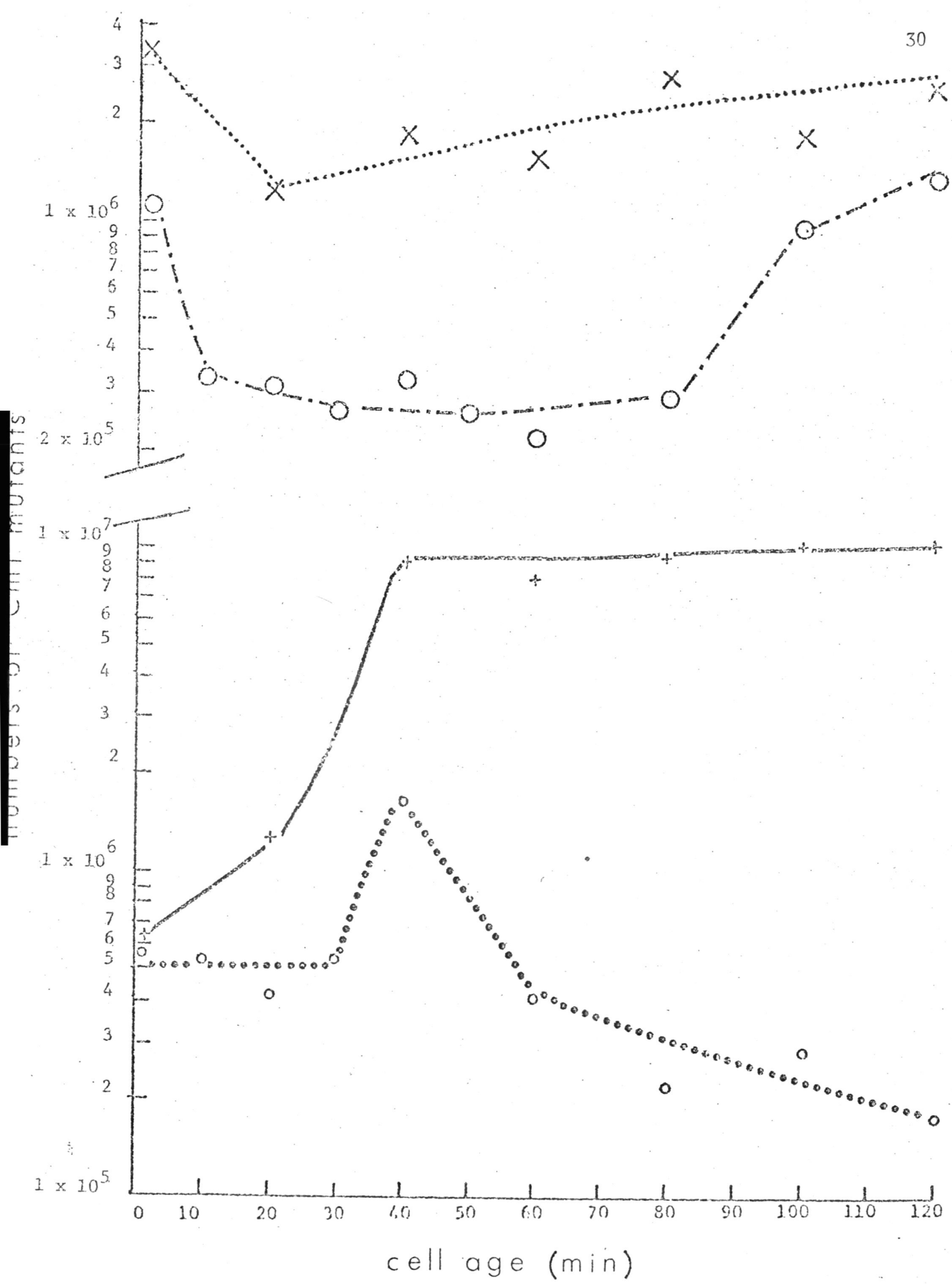


Strain 2 treated with 200 µg NTG per ml in saline

Strain 2 treated with 200 µg NTG per ml in buffer

Strain 7-8 treated with 200 µg NTG per ml in saline

Strain 7-8 treated with 200 µg NTG per ml in buffer

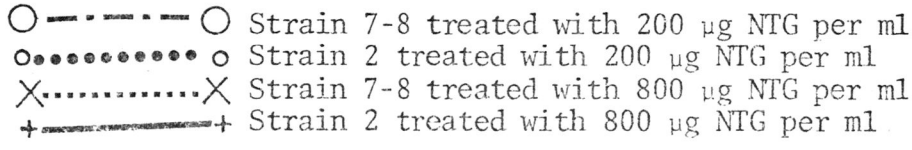

4
3
2
1 x 10^6
9
8
7
6
5
4
3
2 x 10^5
1 x 10^7
9
8
7
6
5
4
3
2
1 x 10^6
9
8
7
6
5
4
3
2
1 x 10^5
0
10
20
30
40
50
60
70
80
90
100
110
120
cell age (min)
numbers of cell mutants

shown in Figure 4. From the plots we see that treatment with NTG (200 µg/ml) in saline caused a drop in the numbers of mutant colonies for strain 2 immediately after the first 10 minutes and a gradual increase in mutant numbers during the remaining time of a single-growth cycle. The numbers of Cml mutants for the same strain dropped immediately after the first 10 minutes of the generation time, remained constant for the following 70 minutes, and sharply increased after 80 minutes when treated with the same concentration of NTG in buffer at pH 5.5. Strain 7-8 showed a sharp increase in numbers of Cml mutants from the beginning of the single-growth cycle for 40 minutes and then remained constant for the remainder of the cycle when treated with NTG (200 µg/ml) in saline. When treated with the same concentration of NTG in a buffer solution (pH 5.5), a peak in the numbers of Cml mutants occurred 40 minutes after release from PEA inhibition followed by a gradual decline throughout the last half of the generation time.

To determine if the concentration of mutagen would affect the apparent replication time for a gene, the cml and mtc loci were determined for the two strains of S. aureus using NTG concentrations of 200 µg/ml and 800 µg/ml at pH 5.5 (Figures 5a and 5b). The replication times for the genes in strain 2 are cml at 120-0 minutes (Figure 5a) and mtc at 10-20 minutes (Figure 5b) for either 200 µg or 800 µg of NTG per ml. The gene replication times for strain 7-8 were cml at 30-40 (Figure 5a) and mtc at 10-20 minutes (Figure 5b) for both 200 µg and 800 µg NTG per ml. Both strains showed a drop in numbers of mutants

Figure 5a. The effect of NTG concentration on the mapping position of the cml locus of S. aureus strains 7-8 and 2

Legend:



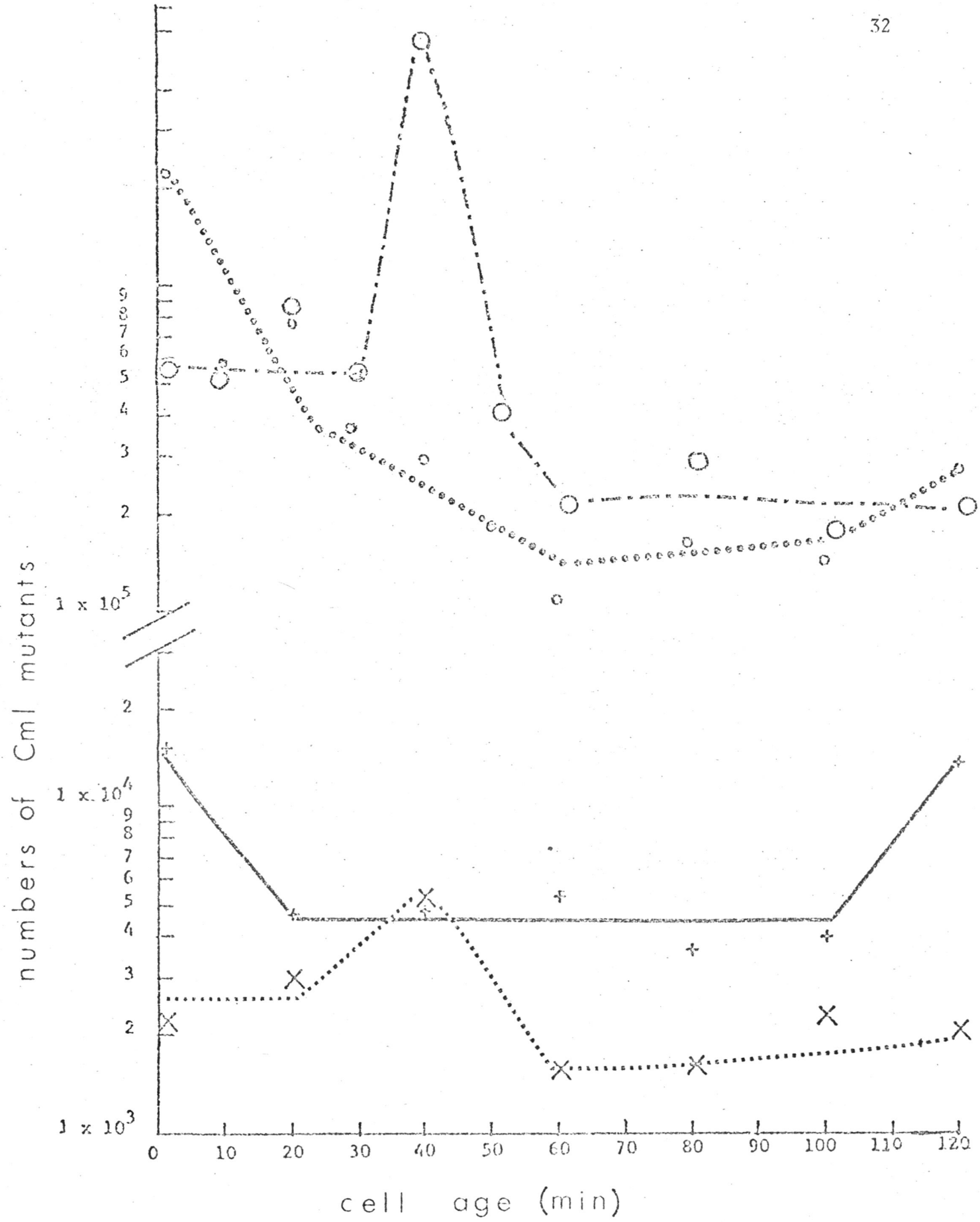
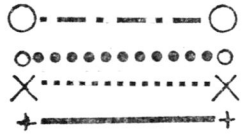

numbers of Cml mutants
1 x 10^5
9
8
7
6
5
4
3
2
1 x 10^4
9
8
7
6
5
4
3
2
1 x 10^3
0
10
20
30
40
50
60
70
80
90
100
110
120
cell age (min)

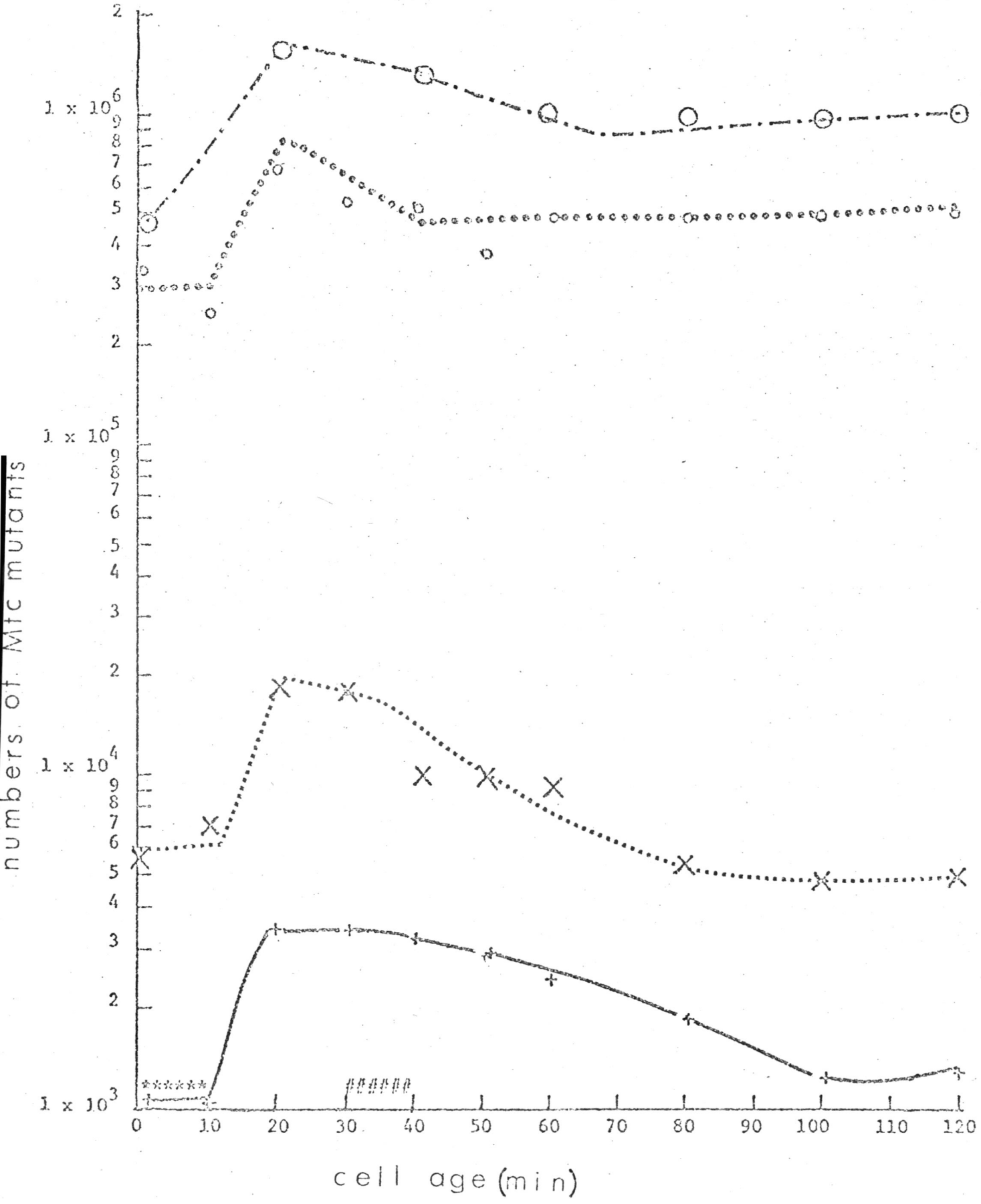
Figure 5b. The effect of NTG concentration on the mapping position of the mtc locus of S. aureus strains 7-8 and 2

Legend:


Strain 7-8 treated with 200 µg NTG per ml
Strain 2 treated with 200 µg NTG per ml
Strain 7-8 treated with 800 µg NTG per ml
Strain 2 treated with 800 µg NTG per ml

Controls: **** Time of cml gene mutation for strain 2

Time of cml gene mutation for strain 7-8


numbers of Mtc mutants
1 x 10^6
1 x 10^5
1 x 10^4
1 x 10^3
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70
80
90
100
110
120
cell age (min)

after the replication point had been passed in the mapping of cml and mtc loci. The results show that treatment with NTG at 800 µg/ml does result in approximately 100-fold decrease in total numbers of mutant colonies obtained during the mapping procedure, but does not change the temporal location of the cml or mtc genes.

The gene locus for cml, as determined by the synchronous chromosomal mapping technique, has been shown not to be affected by either the concentration or the pH of the solution of NTG used. Buffered solutions (pH 5.5) of NTG at 200 µg/ml were employed for mutagenesis in subsequent experiments (Figures 6a, 6b, and 6c). The points of increase in numbers of puromycin (Pym), neomycin (Neo), and streptomycin (Str) mutants are shown in Figures 6a, 6b, and 6c respectively. Each of these figures contains data determined for 2 gene loci, the unknown gene and a previously mapped gene as a reference. The two genes were mapped simultaneously for each culture. Figure 6a shows the temporal location for the pym locus on strains 7-8 and 2. Increase in numbers of Pym mutants for strain 7-8 occurred at 10-20 minutes and for strain 2 occurred at 35-45 minutes. Increase in numbers of Mtc mutants was used as the reference for both strains (Figure 6a). In Figure 6b, strain 7-8 showed increased numbers of Neo mutants after 15-25 minutes and strain 2 after 0-10 minutes. The locations of the mtc and pym genes were used as reference points for strain 7-8 and the location of mtc gene was used as reference for strain 2. Figure 6c shows the times of increase of numbers of Str mutants occurred at 0-10 minutes for strain 7-8 and after 70-80 minutes for strain 2. The time of increase in numbers of Cml mutants was used as a reference marker for both strains.

Figure 6a. Numbers of Pym mutants obtained by sequential mutagenesis during one generation cycle of synchronized cells of S. aureus strains 7-8 and 2

Legend:

○·—·—·—○ Strain 7-8 treated with 200 µg NTG per ml in buffer

○●●●●●●●●●●●○ Strain 2 treated with 200 µg NTG per ml in buffer

Controls: **** Time of mtc gene mutation for strain 2

Time of mtc gene mutation for strain 7-8

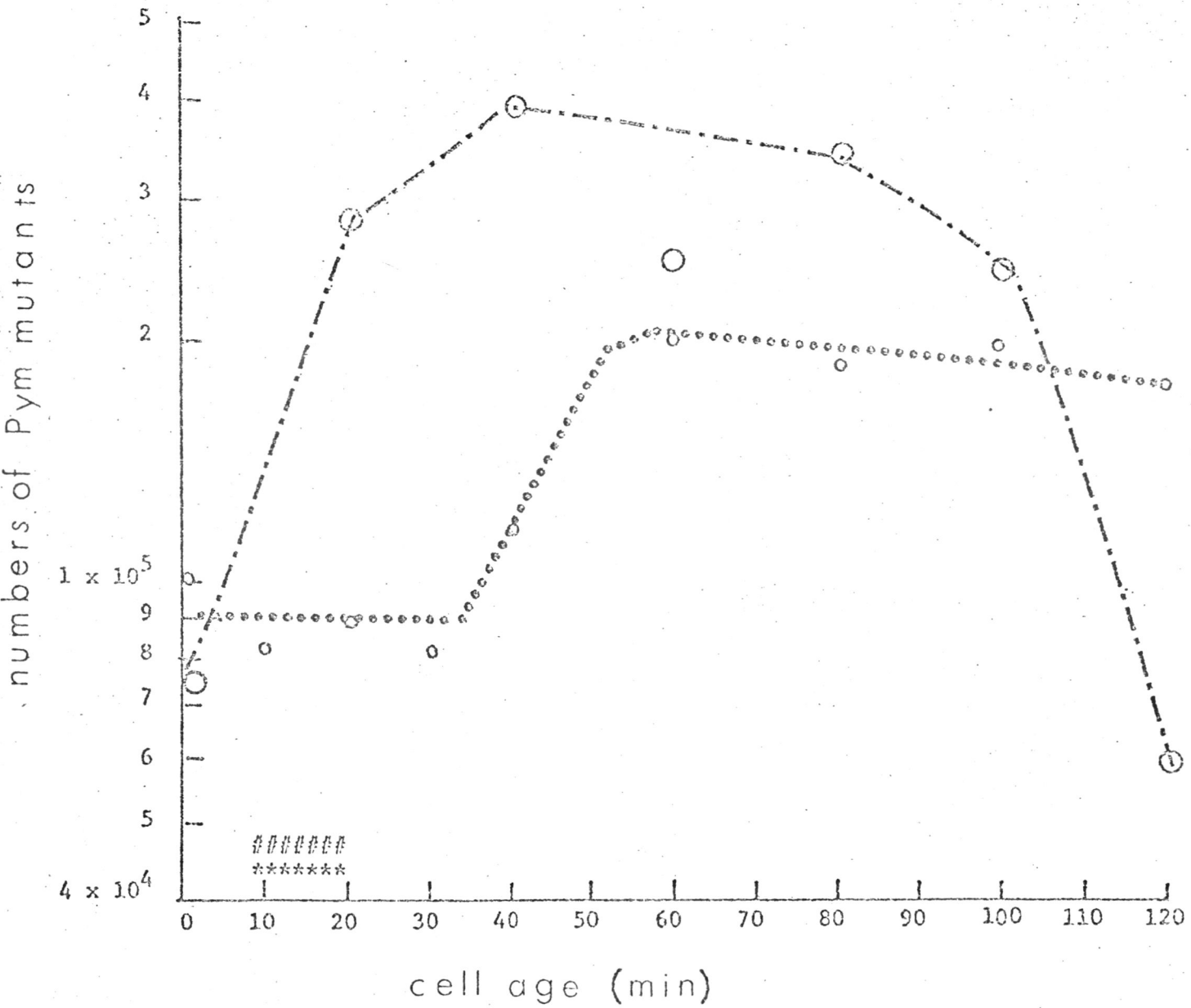

numbers of Pym mutants
5
4
3
2
1 x 10^5
9
8
7
6
5
4 x 10^4
0
10
20
30
40
50
60
70
80
90
100
110
120
cell age (min)

Figure 6b. Numbers of Neo mutants obtained by sequential mutagenesis during one generation cycle of synchronized cells of S. aureus strains 7-8 and 2

Legend:

○·—·—··—○ Strain 7-8 treated with 200 µg NTG per ml in buffer

○••••••••••○ Strain 2 treated with 200 µg NTG per ml in buffer

Controls: **** Time of mtc gene mutations for strain 2

Time of mtc and pym gene mutations for strain 7-8

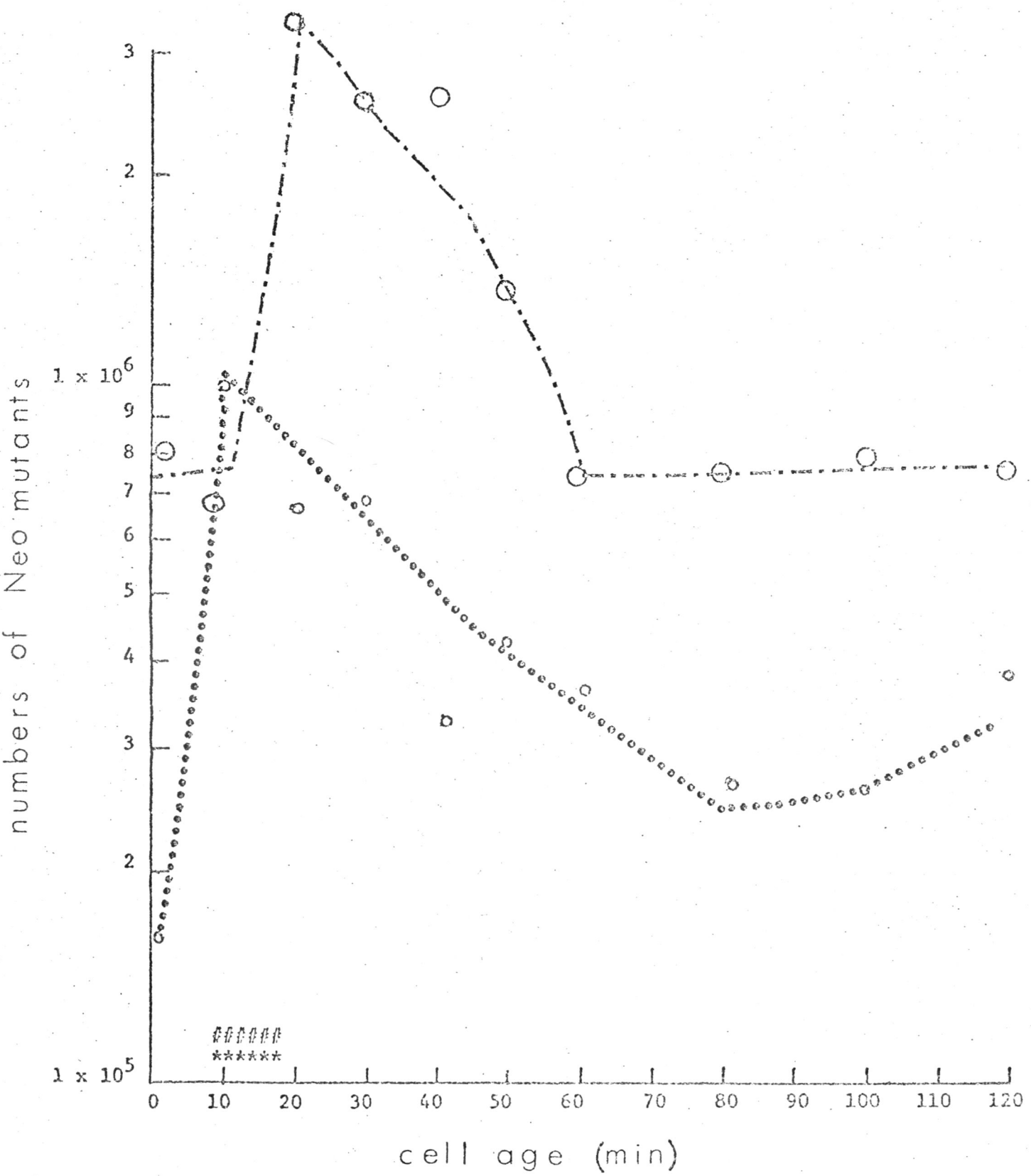

numbers of Neo mutants
3
2
1 x 10^6
9
8
7
6
5
4
3
2
1 x 10^5
0
10
20
30
40
50
60
70
80
90
100
110
120
cell age (min)

Figure 6c. Numbers of Str mutants obtained by sequential mutagenesis during one generation cycle of synchronized cells of S. aureus strains 7-8 and 2

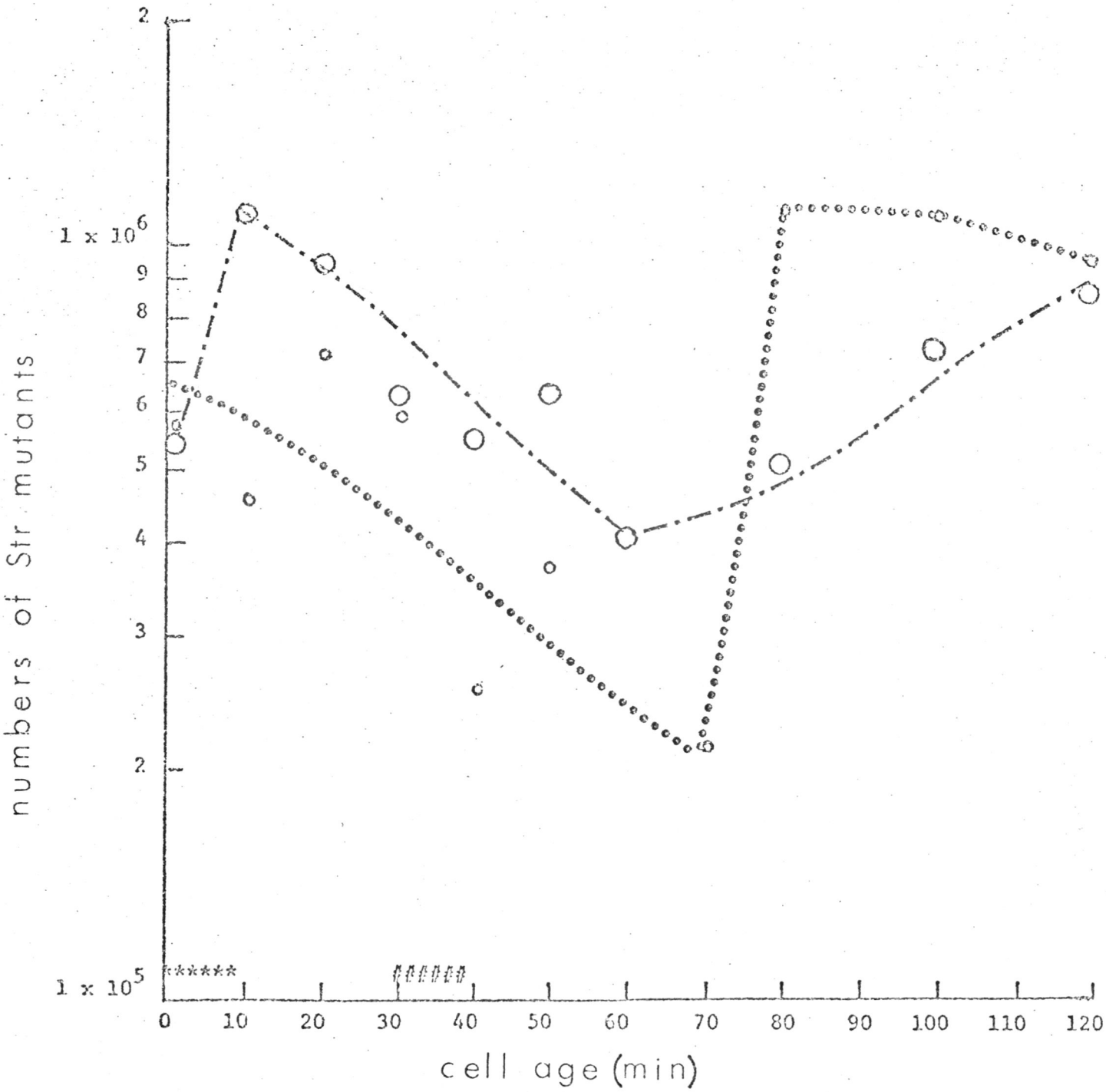
Legend:

○.—.—.—○ Strain 7-8 treated with 200 µg NTG per ml in buffer

○••••••••••○ Strain 2 treated with 200 µg NTG per ml in buffer

Controls: **** Time of cml gene mutation for strain 2

Time of cml gene mutation for strain 7-8


2
1 x 10^6
9
8
7
6
5
4
3
2
1 x 10^5
numbers of Str mutants
******
//////
0
10
20
30
40
50
60
70
80
90
100
110
120
cell age (min)

DISCUSSION

Implicit in the synchronous chromosomal mapping technique of Altenbern (1968) are the assumptions that first a means can be found to synchronize the cultures to be tested, and secondly a means can be found to produce specific mutations in the region of the chromosome being replicated at any specific time in enough of the cells being tested to produce a large number of mutants distinguishable from other cells in the culture. Results in this paper verify synchronization and specific chemical mutation can be produced in Staphylococcus aureus.

Phenethyl alcohol (0.40%) was shown to transiently inhibit DNA synthesis by cultures of S. aureus strains 7-8 and 2 (Figure 1). Assuming that S. aureus has a point of initiation of DNA replication which is controlled in a manner similar to that of E. coli described by Lark and Renger (1969), these PEA-treated cultures of S. aureus should have synchronized chromosomal replication. Comparison of the differences in numbers of Cml-resistant mutants obtained from PEA-treated and untreated cultures of S. aureus strains 7-8 and 2 periodically exposed to NTG during the time of a replication cycle showed the untreated cultures contained a constant number of mutants over the entire testing period while the treated culture showed a peak period of increase in numbers of Cml-resistant mutants (Figure 2). This indicated the cultures were in fact synchronized after release from the 120-minute incubation period in PEA.

The two strains of S. aureus were found to have different viabilities and mutabilities when exposed to the same concentration of NTG (Figures 3a, 3b). These results suggest possible differences in the two strains. First, strain 7-8 could be more permeable to NTG than is strain 2; therefore, the effect on cells of strain 7-8 would be greater. High concentrations of NTG produce more death and correspondingly fewer mutants than low concentrations. Second, there is also the possibility that differences in the base composition of the DNA would explain the different sensitivity of the two strains to NTG. As NTG has been postulated (Sussmuth, Haerlin, and Lingens, 1970; 1972) to react with specific base pairs and specific base sequences in DNA, the DNA of strain 7-8 could be mutated in larger areas by the treatment with NTG than the DNA of strain 2 or react with specific sequences in DNA present in strain 2 producing a lethal effect. Either of these explanations could account for greater numbers of antibiotic-resistant mutants obtained from strain 7-8 as compared with strain 2.

There is no general agreement as to the precise action of NTG on the bacterial chromosome. Cerdá-Olmedo, et al (1968) proposed that NTG caused mutation only at the replication point of the chromosome. Altenbern (1966, 1968) proposed that NTG reacted with the bacterial chromosome causing a doubling in the rate of mutation when the DNA of the gene in question was replicated, and maintained a higher mutation rate of that gene through the completion of the replication cycle. Our results show the locus for Cml resistance remained the same upon treatment with NTG in saline and nutrient media (Figure 2), saline

alone (Figure 4), and in buffered solutions (Figures 4, 5a). With NTG in saline and in saline with nutrient media, the point of replication for the gene was followed by a plateauing of the numbers of Cml mutants. The trend was more evident in strain 7-8 than in strain 2. These results would indicate that NTG not only caused mutation of a specific gene at its replication point but caused a doubling of the numbers of this mutant throughout the remainder of the replication cycle, in agreement with Altenbern (1966, 1968). Treatment of cells with NTG in buffer at pH 5.5 in the absence of nutrients produced narrow peaks of increased numbers of mutants followed by a decrease to the level of mutation prior to the peak of mutagen activity (Figures 4, 5a). Treatment with NTG in buffer at pH 5.5 produced mutation levels that were more consistent than the other treatments from one experiment to another. These results are in agreement with Cerdá-Olmedo et al (1968) that NTG acts only at the chromosomal replication point.

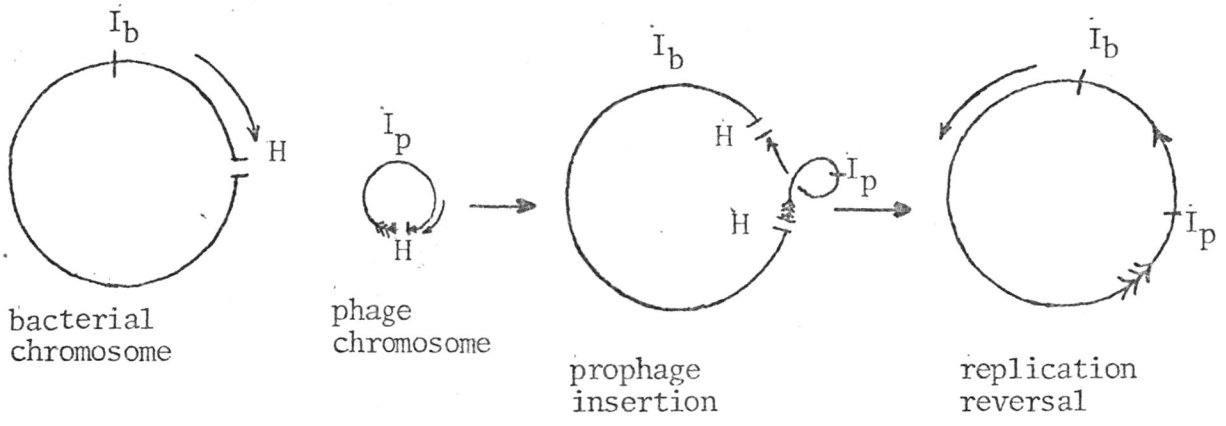
The sustained increased numbers of mutants obtained from treatment with NTG in saline could be caused by the effects of pH on the activity or absorption of the mutagen, or viability of the mutants. Sussmuth and Lingens (1969) reported NTG uptake and mutation rate changed with pH changes from 3.5 to 8.0 in both Escherichia coli B and Saccharomyces cerevisiae with optimum absorbance and mutation rates occurring at pH 6.0-6.5. A test was performed in which non-synchronized cultures of S. aureus strains 7-8 and 2 were treated with NTG (200 µg/ml) in saline solutions adjusted to pH in the range of 4.5 to 8.0 in 0.5 pH increments. The greatest survival occurred at pH 5.5 and 8.0; however,

a larger percentage of Cml-resistant mutants were obtained at pH 5.5 than at pH 8.0. The pH of the solution at the time of treatment caused as much as a 20-log change in the numbers of viable cells surviving treatment. Llovers et al (1973) stated the optimum pH for survival of NTG treatment in E. coli is pH 5.5.

Comparative studies of the cml and mtc loci of strains 7-8 and 2 revealed the time of replication of these genes was independent of NTG concentration although there was a 2.5-log reduction in numbers of mutants recovered with high (800 µg/ml) concentration (Figures 5a, 5b). Determination of a locus of a gene occurring in the 0.1% survivors (mostly mutants) of a total population might have no relation to the position of the gene on a normal (non-mutated) cell's chromosome. If this were the case, employing two different concentrations of mutagen would likely produce different mapping positions for the same gene depending on the amount of chromosomal change which the particular concentration produced. Since the apparent gene locus for cml and mtc did not vary with the concentration of NTG employed for mutagenesis, the credability of the synchronous chromosomal mapping technique was affirmed. Since high concentrations of NTG produce fewer survivors and therefore fewer numbers of mutants, the use of the highest concentration of NTG that produces mutation without altering the apparent position of the gene simplifies the mapping technique and reduces the possibility of error.

A linear map comparing the replication times of five antibiotic-resistant loci for S. aureus strains 7-8 and 2 is shown on page 44. The

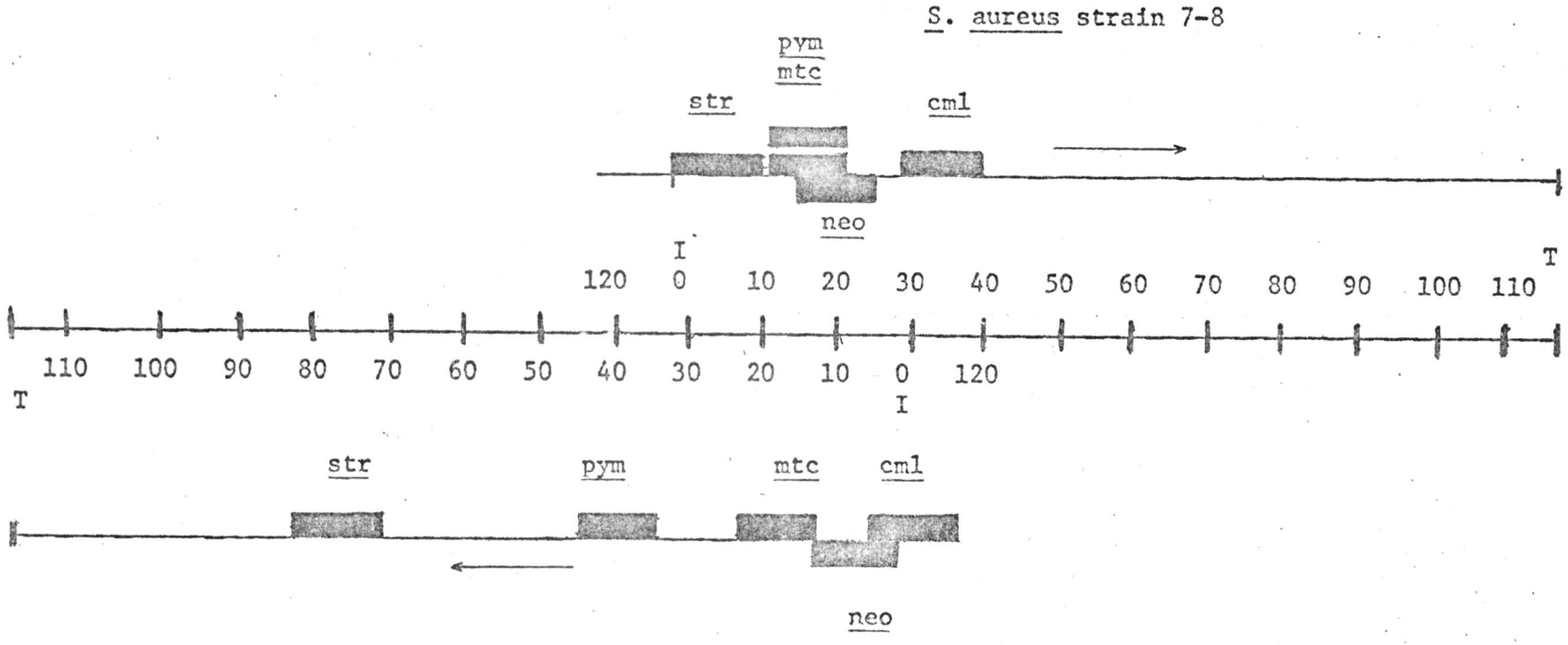
point of initiation of chromosomal synthesis for strain 7-8 was displaced approximately 1/4 the total distance of the replication map from the point of initiation of chromosomal replication by strain 2. Assuming the orders of genes on the chromosomes in the two strains are the same, chromosomal replication in strain 7-8 occurred in the opposite direction from chromosomal replication in strain 2. This is not consistent with Altenbern's (1969, 1973a) findings. The difference in the apparent direction of chromosomal replication of S. aureus strain 7-8 as well as the difference in the point of initiation of strains 7-8 and 2 could be due to the presence of prophage in the chromosome of strain 2. Numerous workers have reported the great majority of S. aureus strains of human origin carry temperate bacteriophages (Rountree, 1949; Gorril and Gray, 1956; Blair and Carr, 1961). Therefore, the probability exists cultures of S. aureus isolated from humans would likely be lysogenic. Similarly, Pulver, Pillich, Klein, and Kriv'ánková (1974) found that all human pathogenic strains of S. epidermis isolated from human carriers carried temperate bacteriophages with at least 30% carrying two phages. S. aureus strain 7-8 of bovine origin has been shown by experiments with UV, mitomycin C, and acriflavin treatments (inducing and curing agents) to be non-lysogenic (unpublished data). The insertion of a prophage into the chromosome of S. aureus could cause a change in the direction of chromosomal replication as indicated in the following diagram. The mechanism of insertion was proposed by Campbell (1962).



Key: I_p = point of initiation of phage chromosomal replication
I_b = point of initiation of bacterial chromosomal replication
H = regions of base homology
Arrow indicates direction of replication

If the prophage was inserted into the chromosome of the bacterium in such a manner that the prophage chromosome replicated in the opposite direction to that of the host chromosome, the following possibilities exist. First, if the prophage contained a repressor gene the product of which could inhibit the initiation site of the bacterial chromosome, the prophage site of initiation of chromosomal replication could serve to initiate the replication of the chromosomal complex and the direction of replication would be opposite the direction of replication of the chromosome of non-lysogenic cells. This could explain the difference in direction of chromosomal replication by strains 7-8 and 2. Additionally, prophages would likely be inserted into a specific area of the host chromosome because of a base homology. Therefore all strains lysogenized by this phage in this manner would have the prophage added at the same point. Any effect of prophage on sequence and distances between genes on the chromosome would be uniform from culture to culture. This would explain the results of Altenbern (1969, 1971a, 1973a). Also, if prophage replicated at a

Comparison of the loci of genes and direction of chromosomal replication for Staphylococcus aureus strains 7-8 and 2



S. aureus strain 2

Legend: Streptomycin (str); puromycin (pym); mitomycin C (mtc); chloramphenicol (cml)
I = point of initiation of chromosomal replication
T = point of termination of chromosomal replication
Arrow indicates the direction of chromosomal replication

different rate from the remainder of the chromosome a shifting of the total genomic map such as proposed by Altenbern (1969) in the MRF strains would occur. Secondly, certain strains of S. aureus could contain an initiator site insensitive to repression by the prophage resulting in two active initiator sites per chromosome. The chromosomal map obtained from these cells would show the presence of two replicons. This could account for the existence of the two replicons proposed for some strains of S. aureus by Altenbern (1967) and could explain the gene-shifting effect noted by Altenbern (1971a). As stated earlier, in either type of prophage insertion, there would be a specific area in the host chromosome where the phage would be most likely to insert. Insertion of the prophage at the 30- to 40-minute period of the normal chromosomal replication cycle could produce the difference in initiation site noted in strain 2.

The order of the mapped genes for the two strains was identical and there was excellent agreement of the distance between genes for the mtc, neo and cml loci. However, the distances between the str, pym and mtc loci are much shorter on the chromosomal map of strain 7-8 than in strain 2. These latter genes all appear close to the point of initiation of chromosomal replication of strain 7-8, while in strain 2 the pym and str loci are separated and located more toward the terminus of the replication map. Altenbern (1969) found the duplication time of a specific locus in a single strain of S. aureus could vary as much as 10 minutes depending on the lot of medium used when mapping. This variation increased the further the gene site was from the point of

initiation of chromosomal replication. Similar factors may have been responsible for the difference obtained in the position and distance between the pym and str loci for strains 7-8 and 2. All genes were mapped in individual experiments and the distances between two genes occurring late in the replication cycle could possibly vary as much as 20 minutes in the time of replication compared to another strain in which these genes mapped closer to the origin without indicating any real difference in actual chromosomal distance between the genes.

All loci we mapped for strain 2 agreed with Altenbern's data (1969, 1971b) obtained by synchronous chromosomal replication and marker frequency analysis except the marker for puromycin resistance. Altenbern (1971b) mapped the position of Pym resistance by marker frequency analysis. Interpolation of the data supplied by Altenbern (1971b), revealed the puromycin locus should have been located near the origin of the chromosomal map of strain 2. We found the location of the gene for Pym resistance of strain 2, determined by synchronous chromosomal mapping, occurred at 35-45 minutes on the replication map. The reason for this apparently large variation in the position of the pym marker obtained by the two mapping techniques is unknown. A similar difference in mapping of the tryptophan locus in strain "Maybush" was described by Altenbern (1971a).

Mapping of the genes for acriflavin, penicillin, and tetracycline resistance for strains 7-8 and 2 were attempted using NTG in saline solution with variable and inconclusive results. Altenbern (1971a) found that concentration of acriflavin was critical in mapping this

gene in strain 2. A single concentration of acriflavin permitting mapping of this gene in strains 7-8 and 2 was not obtained. Strain 7-8 was shown to be extremely sensitive to penicillin and concentrations of less than 0.5 unit/ml inhibited growth of mutagen-treated cultures completely. Alternately, strain 7-8 was found to have greater tolerance to tetracycline than strain 2, requiring different concentrations of that antibiotic to be employed in the selective medium. This complicated the procedure and we felt the results of these experiments were inconclusive.

The difference in direction and point of initiation of chromosomal replication in the two strains tested may indicate distinct genetic differences in S. aureus strains of animal origin and human origin. Numerous strains of human origin have been mapped and differences in the strains have been small, while S. aureus strain 7-8 is the only strain of animal origin to have been mapped up to this time. Strain 7-8, however, is a non-lysogenic strain, and although no direct evidence has been given to indicate that the strains of human origin are lysogenic, the accumulated data on strains of human origin suggests that they most likely would be lysogenic. Until the chromosomes of lysogenic strains of S. aureus from animal origin and known non-lysogenic strains of human origin are mapped by sequential chromosomal mapping, the chromosomal changes found in strain 7-8 cannot be explained.

SUMMARY

1. Genomic maps of Staphylococcus aureus strains 7-8 and 2 were obtained using the synchronous chromosomal mapping technique.
2. Phenethyl alcohol was found to inhibit DNA synthesis and release from phenethyl alcohol inhibition produced a population of cells synchronously replicating their chromosomes.
3. Varying the conditions of nitrosoguanidine mutagenesis did not change the position on the replication maps for a specific locus in synchronized populations. The pH of the solution of mutagen was found to affect the numbers of mutants obtained after the gene in question had replicated.
4. The point of initiation and the direction of chromosomal replication were different for the two strains of S. aureus tested. Prophage insertion was proposed as one explanation for these differences.
5. The sequence of genes on the chromosomes of both strains was the same. Genes replicating early in the replication cycle of both strains had similar mapping distances between genes while those genes mapping near the origin in strain 7-8 mapped closer together than the same genes located near the terminus in the strain 2.

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