James Van Taylor III. COMPARATIVE INTRAGENIC MAPPING OF ASPARTIC-ACID AUXOTROPHS OF STAPHYLOCOCCUS AUREUS USING TRANSDUCTION AND TRANSFORMATION. (Under the direction of Wendall E. Allen) Department of Biology, ECU, Greenville, N. C., July 1976.

Twenty-nine DNase$^-$ auxotrophic strains obtained after mutagenic treatment of the Staphylococcus aureus parent strain 7-8 with N-methyl-N´-nitro-N-nitrosoguanidine were selected for use in fine-structure genetic analysis. The auxotrophs were divided into two groups, those that showed evidence of reversion, probably representing point mutations, and those that showed no evidence of reversion, probably representing deletion or multisite mutations. Auxotrophs having point mutations were proposed to have chromosomal defects within the same genetic locus as determined by the lack of syntrophism.

The auxotrophs having point mutations were tested for lytic sensitivity to the serological group B staphylococcal typing phages 52, 53, 29, 79, 80, 81 and phage Ø11. Auxotrophs and their respective lytic phages were designated for possible use in transductional studies. A transductional screening test was used to identify transductionally competent phage-auxotroph groups for use in fine-structure genetic analysis. Each of the staphylophages showed competency in transducing some portion of the auxotrophs.

The aspartic-acid-deficient auxotrophs, Asp-$^-$4, Asp-$^-$7, and Asp-$^-$11, were selected for transductional analysis using typing phage 80 and phage

Ø11 and for transformational analysis. Lysogeny was shown not to be a requisite for the aspartic-acid transductions. Transductional frequencies of the phage Ø11-lysogenized mutants [Asp-$^{-}$4(Ø11), Asp-$^{-}$7(Ø11), and Asp-$^{-}$11 (Ø11)] using typing phage 80, were lower than the transductional frequencies using the non-lysogenized auxotrophs and the same phage. Phage Ø11 was not as effective a transducing phage for the non-lysogenized auxotrophs as typing phage 80.

Intragenic maps of three aspartic-acid mutation sites on the chromosome of S. aureus were prepared from data obtained by three methods: reciprocal-transduction analysis using typing phage 80 and phage Ø11, recipient-capacity determination using typing phage 80, and transformational analysis of the phage Ø11-lysogenized mutants. There was agreement in the order and relative position of the three aspartic-acid mutation sites determined from results of reciprocal-transduction experiments using typing phage 80 and recipient-capacity experiments. Results of preliminary experiments for both transductional analysis with phage Ø11 and transformational analysis of these mutants were inconclusive.

COMPARATIVE INTRAGENIC MAPPING OF ASPARTIC-ACID AUXOTROPHS OF *STAPHYLOCOCCUS AUREUS* USING TRANSDUCTION AND TRANSFORMATION

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James Van Taylor III

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OF ASPARTIC-ACID AUXOTROPHS OF

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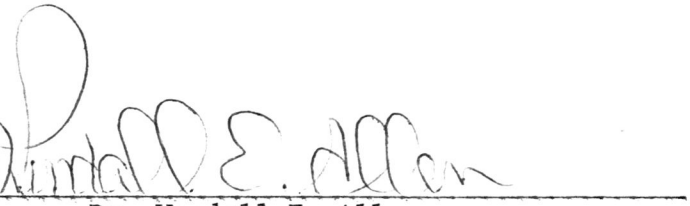
SUPERVISOR OF THESIS 

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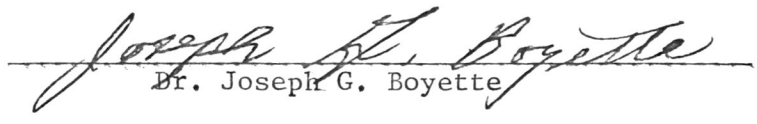

Dr. Joseph G. Boyette

Dedicated

to

My Wife, Pat

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TABLE OF CONTENTS

PAGE

LIST OF TABLES

INTRODUCTION

Our knowledge of the genomic organization of Staphylococcus aureus has remained limited despite the availability of generalized transduction in this species since 1959 (Morse, 1959). Intergenic analyses of several amino-acid biosynthetic pathways have been reported (Ritz and Baldwin, 1962; Kloos and Pattee, 1965; Humbert and Baldwin, 1963; Smith and Pattee, 1967; Barnes et al. 1971). Much more is known about the genetic constitution and behavior of plasmids of S. aureus (see, Ritz and Baldwin, 1958, 1961; Harmon, 1966; Harmon and Miller, 1967; Rudin and Lindberg, 1975; Schwesinger and Novick, 1975; Novick et al. 1976). There are no reports of intragenic studies with S. aureus.

Transformation of staphylococci became possible with the discovery of the enzyme lysostaphin in 1960 by Schindler and Schuhardt providing a method for dissolution of the tough cell wall covering releasing biologically-active DNA, and the description in 1972 by Lindberg et al. of the conditions under which a culture of S. aureus became competent to take up DNA from its surroundings. Transformation, shown to depend on the presence of the ϕ11 prophage in the recipient and exposure of cells to calcium ions at a particular stage of growth, has now been reasonably well characterized (Rudin et al. 1974; Sjostrom et al. 1972; Sjostrom and Philipson, 1974). Recently, three genetic linkage groups demonstratable by transformation have been reported on the chromosome of S. aureus (Pattee and Nevelin, 1975).

Seemingly, all of the technical problems preventing the fine-structure intragenic mapping of mutation sites in S. aureus have been solved. What is lacking in the literature is a comparative study mapping mutation sites by transductional and transformational genetic analysis. The purpose of this investigation was to 1) obtain a collection of DNase$^-$ S. aureus strains containing point mutations producing the same auxotrophic phenotype after mutagenic treatment of the S. aureus parent strain 7-8, 2) identify transductionally-competent phage-auxotroph groups, and 3) determine comparative intragenic maps of selected mutation sites from data obtained by three methods: reciprocal-transduction analysis, recipient-capacity determination, and transformational analysis.

LITERATURE REVIEW

Recovery and Characterization of Bacterial Auxotrophs

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) has been a commonly employed mutagen for bacteria. It was first used to induce mutations in Escherichia coli by Mandell and Greenberg (1960), and further characterized by Adelberg, Mandel, and Chen (1965). NTG acted as a powerful mutagen under conditions affording high survival of treated bacteria, and it apparently functioned as an alkylating agent (Cerdá-Olmedo and Hanawalt, 1967), particularly at the fork of DNA replication (Cerdá-Olmedo, Hanawalt, and Guerola, 1968), and produced single-strand breaks in the DNA (Olson and Baird, 1969). It has been used to induce auxotrophic (biochemically deficient) mutants from prototrophic (nutritionally complete or "wild-type") staphylococci by Altenbern (1967 a,b), Barnes, Bondi, and Moat (1969), Hengstenberg et al. (1969), Omenn and Friedman (1970), Proctor and Kloos (1970), Lindberg et al. (1972), and Pattee and Neveln (1975).

The isolation of auxotrophic mutants has been expedited by the "penicillin selection method" of Davis (1948), involving the selective inhibition of prototrophs growing in minimal medium containing prototrophic and auxotrophic cells, and the "replica-plating technique" of Lederberg and Lederberg (1952), permitting the identification of auxotrophic colonies among prototrophic colonies growing on complete medium. Application

of these techniques enhanced the number of auxotrophic cells present and facilitated their recovery from a mixed population of prototrophs and auxotrophs. Clowes and Hayes (1968) reported a method affording identification of the specific growth requirement(s) of auxotrophs by determining their ability to grow on minimal medium supplemented with various combinations of amino acids, vitamins, and purine and pyrimidine bases.

Transduction

Zinder and Lederberg (1952) described a means of genetic transfer in bacteria by bacteriophages which they termed transduction, subsequently defined as phage-mediated transfer of genetic material from one host to another (Bertani, 1955).

Two types of transduction have been defined, generalized and specialized, according to the range of bacterial markers available for transmission in a given transducing system. Typical examples of the generalized type were transductions performed by phage P22 in Salmonella typhimurium (Zinder and Lederberg, 1952) or phage P1 in E. coli (Lennox, 1955), in which almost any marker of a donor strain was transduced. In specialized transduction, a specific phage was shown to transduce only specific markers, such as the gal (galactose) loci transduced by phage lambda in E. coli K-12 reported by Morse, Lederberg and Lederberg (1956). Phage Ø80 in E. coli has been another phage-host system demonstrating specialized transduction (Matsushiro, 1963).

Transduction was first described in staphylococci by Cavallo and Terranova (1955). Transduction has been reported in staphylococci of both plasmidal markers [penicillinase production (Ritz and Baldwin, 1958, 1961; Harmon, 1966; Harmon and Miller, 1967; Rudin and Lindberg, 1975), erythromycin resistance (Novick, 1967), streptomycin and erythromycin resistance (Yu and Baldwin, 1971)] and chromosomal markers [threonine (Edgar and Stocker, 1961), tryptophan (Ritz and Baldwin, 1962), methionine (Humbert and Baldwin, 1963 and Harmon and Miller, 1967), uracil, riboflavin, guanine (Carere and Spada-Sermonti, 1964), histidine (Kloos and Pattee, 1965), isoleucine-valine (Smith and Pattee, 1967), and lysine (Barnes et al. 1971)].

Relationship of Serological Group and Transducing Capacity of Staphylococcal Bacteriophages

Staphylococcal phages have been serologically grouped by Rountree (1949) and Rippon (1956). The recommended phage-typing set contained phages of serological groups A, B, F, and L according to Blair and Williams (1961). Since staphylococcal transduction was first reported by Cavello and Terranova (1955), transducing ability has been found for a number of staphylophages, all, however, belonging to serological group B or related serological group F phages (Pattee and Baldwin, 1961; Dowell and Rosenblum, 1962a; Novick, 1967). Infectivity of serological group B staphylococcal phages was shown to be almost completely lost

(90-98%) upon treatment with sodium citrate (Rountree, 1951).

Reports of generalized transduction with serological group B typing phages included Morse (1959), Korman (1960), Edgar and Stocker (1961), and Barnes et al. (1971) with typing phage 53, Ritz and Baldwin (1961, 1962) and Carere and Spada-Semonti (1964) with typing phage 80; Pattee and Baldwin (1961) with typing phages 29, 79, and 52A; Kloos and Pattee (1965) and Smith and Pattee (1967) with typing phage 83; and Ritz and Baldwin (1958) with typing phage 52. One serological group B phage, Ø11, not in the typing series, has been characterized as a generalized transducing phage (Novick, 1963).

Novick (1967) described a specialized transducing fragment (Ø11de) obtained from staphylophage Ø11. The particle was composed of a part of the phage Ø11 chromosome and that part of the penicillinase plasmid containing the erythromycin-resistance marker. Recently Rudin and Lindberg (1975) reported a second specialized transduction system. Their staphylophage (Ø14) differed from phage Ø11 only in its immunity locus, and specifically transduced the penicillinase plasmid at high frequency (10^{-1}).

Nature of the Transducing Particle

The characteristics or features of the exterior of transducing phages (size, shape, adsorption characteristics, and serological properties) were indistinguishable from those of normal particles of the phage concerned (Zinder, 1953; Stocker, 1958). They differed from normal phage

particles in that they carried the chromosomal DNA of the host bacterium instead of all or some viral DNA (Ikeda and Tomizawa, 1965). Typing phage 80 DNA had a molecular weight of 3.1×10^7 (Pariza and Iandola, 1974), composed of approximately 7.75×10^4 nucleotides.

Two models accounting for the inclusion of a small piece of bacterial chromosome within a phage coat to form a transducing particle have been proposed. Campbell's model (1964), the "hybrid formation mechanism", accounted for specialized transducing capacity of coliphage lambda. The transducing particle contained a "hybrid" DNA molecule consisting of part phage genome and part bacterial genome. Ozeki and Ikeda (1968) proposed the "wrapping choice model" to account for generalized transducing activity. This model proposed a fragment of bacterial chromosome was wrapped accidently by an ordinary phage coat. This transducing phage contained no phage genes.

In generalized transduction, any genetic marker of a donor bacterium was transduced, and consequently, the entire bacterial genome was carried within the lysate (different parts of the genome were carried by different transducing particles). Ozeki (1959) reported probable homogeneity in the size of the fragment of chromosome contained by transducing particles for the P22-*Salmonella* system. Phages capable of transducing histidine markers in *S. aureus* were shown to have homogeneous chromosome fragments by Kloos and Pattee (1965) and Pattee et al. (1968) mostly terminating

at a preferential (particular) point. However, Roth and Hartman (1965) and Pearce and Stocker (1965) reported evidence that genomic fragments in transducing phages were not uniform and some degree of heterogeniety existed among their transducing particles.

Two types of effect following transduction were reported in a series of papers by S. E. Luria and his collaborators. Depending upon the degree of genetic homology between the donor and recipient of the transduced bacterial genes, either integration or lysogenization (latent infection with bacteriophage) occurred. Integration (recombination) occurred if there was high genetic homology and lysogenization occurred due to low genetic homology (Luria et al. 1958; Luria, Adams, and Ting, 1960; Boice and Luria, 1961; and Franklin and Luria, 1961).

A transducing element unable to multiply and with little integrating capacity produced mainly abortive transduction (unilinear inheritance of the non-replicating transducing fragment by a single member of a bacterial population). Abortive transduction was first observed of motility characters in Salmonella (Stocker, Zinder, and Lederberg, 1953). It has been observed for nutritional markers and abortively transduced colonies appeared as "minutes", slowly growing colonies on unsupplemented minimal media (Ozeki, 1956). The phenomenon was considered to be rather common, and has been utilized for complementation studies (Demerec and Ozeki, 1959).

Dowell and Rosenblum (1962b) reported almost all of the novobiocin-resistant transductant clones of staphylococci were lysogenic for the transducing phage under conditions permitting superinfection (high

multiplicity of infection). If superinfection were prevented by addition of sodium citrate, the transductants were nonlysogenic. These results implied the staphylococcal transducing particles were usually defective, and most, if not all of the fragments were unable to lysogenize the transduced cells. Defective (lacking some normal phage functions) transducing particles were also shown with coliphage P1 (Luria, Adams, and Ting, 1960). Further evidence that lysogeny was not a corequisite for staphylococcal transduction was shown by Ritz and Baldwin (1961) and Dowell and Rosenblum (1962a) in reports that typing phage 80, previously characterized as a nonlysogenizing variant of typing phage 52A by Rountree (1959) and Comtois (1960), was capable of transduction.

In staphylococcal transducing systems for chromosomal markers, ultraviolet irradiation of the transducing phage preparation before its addition to recipient bacteria has been routinely used. UV irradiation supposedly acted by enhancing the recombination potential of the fragment with the recipient chromosome (Egan, 1972). Enhanced transduction rates of nutritional markers with UV-irradiated typing phage 80 was reported by Carere and Spada-Sermonti (1964). Barnes et al. (1971) reported transduction of lysine markers of S. aureus using typing phage 53 which had been 99% lytically inactivated by UV-irradiation.

Transformation

Bacterial transformation was discovered in 1928 by Griffith. Transformation has been characterized as a process involving the penetration

of a fraction of the donor's total DNA, obtained by chemical extraction or natural cell lysis, into a related bacterial cell, and replacement, by a process of recombination, of a specific nucleotide sequence in the recipient's genome. If the newly integrated genetic fragment differed from the replaced nucleotide sequence of the recipient, new information or phenotypic change resulted in the transformed cell and its progeny. The molecular weight of transforming preparations of pneumococcal DNA was estimated to be about 5×10^6 (Fluke et al. 1952), composed of approximately 1.25×10^4 nucleotides.

The occurrence of transformation has now been confirmed in a considerable number of bacterial genera and species (review: Spizizen, Reilly, and Evans, 1966). Only recently has transformation been observed in S. aureus (Nomura et al. 1971). According to Lindberg et al. (1972), the main obstacle to demonstrating transformation in S. aureus was in the extraction of high molecular weight, biologically active DNA which was resistant to the activity of staphylococcal deoxyribonuclease. A method designed to isolate S. aureus mutants lacking extracellular nuclease activity was developed by Omenn and Friedman (1970). According to these workers, the possibility of successful transformation with S. aureus could be increased by the use of a nuclease-deficient mutant of S. aureus as a recipient strain.

Because transformation represented an extremely powerful tool in biochemical genetic analysis, many attempts at demonstrating transformation in S. aureus have been made. Little success was realized at first;

Blobel (1961) reported some success in preliminary experiments, and Klesius and Schuhardt (1968) reported no success with several DNA preparations. In 1968, Dobrzánski, Osowiecki, and Jagielski demonstrated intergeneric transformation of streptomycin resistance using Staphylococcus as donor and Streptococcus as recipient.

A stumbling block in demonstrating transformation in S. aureus has been the inability to rupture the thick, rigid staphylococcal cell wall and isolate biologically active DNA. Physical techniques required to disrupt staphylococcal cells were so destructive that the DNA isolated had lost all genetic function (Hayes, 1968). A mild method for releasing DNA from staphylococci was provided by Schlinder and Schuhardt (1964, 1965) who discovered and characterized a staphylolytic enzyme, lysostaphin. Riggs and Rosenblum (1969) reported successful transfection (the uptake by competent recipients of DNA extracted from viruses previously grown in donor cells) using lysostaphin-induced spheroplasts as recipients for phage DNA.

The first reports of S. aureus transformation were limited to resistance markers like streptomycin and metal ions (Imshenetiskii et al. 1959; Imshenetskii and Perova, 1959; Lambina, 1961, Komarov, 1962; Smirnova et al. 1969). Lindberg et al. (1972) described methods of transformation in S. aureus, using erythromycin, penicillin, and cadminum-resistant plasmidal characters and the chromosomal markers thymine and cytosine. They used transforming DNA prepared by phenol extraction of lysostaphin-lysed cells.

Nomuya et al. (1971) demonstrated induction of hemolysin synthesis, a chromosomal marker, by transformation in S. aureus. Recently, Pattee and Neveln (1975) reported several instances of apparent genetic linkage determined by transformation in S. aureus. Ten distinct loci on the chromosome (one involving novobiocin resistance and nine associated with biosynthetic activities) comprising three linkage groups were investigated. The biosynthetic markers studied included thymine, lysine, tryptophan, threonine, histidine, uracil, adenine, and isoleucine.

A recurrent problem in staphylococcal genetics was to determine whether a genetic character was coded by a chromosomal or plasmidal gene. Lindberg and Novick (1973) reported an application of transformation which differentiated plasmidal from chromosomal markers. They found that only plasmidal and not chromosomal markers could be transformed by circular-duplex DNA.

Sjostrom et al. (1973) reported the times during growth of a culture at which competence occurred (the capacity of a bacterium to take up DNA from its surroundings) differed between the wild-type staphylococcal strain (8325N) and a nuclease-deficient mutant (8325 nuc). The wild-type strain showed maximum DNA uptake in the early exponential growth phase where no extracellular nuclease activity was observed. The nuclease-deficient strain showed an additional competence maxima later in the exponential growth phase. Calcium ions were found to be necessary for competence expression.

Rudin et al, (1974) conducted a study concerned with the genetic and physiological factors which influenced transformation of S. aureus (strain 8325). A defined synthetic medium was developed and used for isolation of amino-acid requiring mutants and as a selective medium in transformation experiments. They reported the optimal pH and temperature for transformation were 6.75 to 7.00 and 30 C, respectively, and that calcium ions were required. These conditions were found to be necessary for transformation of both plasmidal and chromosomal markers.

Genetic Mapping

Several techniques of genomic or chromosomal mapping of S. aureus have been reported. The synchronous-chromosomal replication method (Altenbern, 1966, 1968, 1969), the marker-frequency analysis mapping (Altenbern, 1971), and a combination of the two methods (Altenbern, 1971), have been used to map 26 loci on the S. aureus chromosome.

Studies of intergenic relationships have also been reported in S. aureus. Through biochemical studies and transductional analysis of auxotrophs, workers have been able to elucidate several amino acid-biosynthetic pathways in S. aureus. (Ritz and Baldwin, 1962; Kloos and Pattee, 1965; Humbert and Baldwin, 1963; Smith and Pattee, 1967; Barnes et al, 1971).

Intergenic analysis has been demonstrated by Hartman (1956) in tests with Salmonella auxotrophs. By reciprocal transductional analysis,

he constructed a linkage map of the histidine region of the Salmonella chromosome. Hartman et al. (1960) reported an intragenic linkage map of 15 mutant sites within one histidine locus by transductional analysis. Similar studies were reported by Clowes (1960) with Salmonella. Comparable studies have been demonstrated with the tryptophan synthetase system (Yanofsky, 1960) and the structural gene for exonuclease (White et al. 1976) in Escherichia coli. We have been unable to find results of intragenic mapping of mutant sites for S. aureus.

MATERIALS AND METHODS

Cultures

The parent culture of Staphylococcus aureus 7-8 used throughout this study was isolated from the infected teat of a cow with mastitis. The culture lacking the ability to produce DNase ($DNase^-$) was isolated after mutagenic treatment of strain 7-8. Auxotrophs were isolated from the $DNase^-$ strain following mutagenic treatment.

Seven bacteriophages were used in this investigation. Staphylococcal phages 29, 52, 53, 79, 80, 81 were of the International Phage Typing Series. Phage Ø11 was provided by Dr. Richard Novick, Public Health Research Institute of the city of New York, New York.

Media and Buffer

Trypticase Soy Broth (TSB) and Trypticase Soy Agar (TSA, Baltimore Biological Laboratories, BBL, Baltimore, Maryland) were the growth and maintenance media used. Additions to these media produced the following special media:

Designation	Ingredients
TSA + YE	Trypticase Soy Agar + 0.5% (w/v) Yeast Extract (BBL)
TSA + YE + Ca	Trypticase Soy Agar + 0.5% (w/v) Yeast Extract + 400 µg/ml $CaCl_2$
TSB + YE	Trypticase Soy Broth + 0.5% (w/v) Yeast Extract
TSB + YE + Ca	Trypticase Soy Broth + 0.5% (w/v) Yeast Extract + 400 µg/ml $CaCl_2$
TSB + YE + Cit	Trypticase Soy Broth + 0.5% (w/v) Yeast Extract + 0.5% (w/v) Sodium Citrate

The synthetic minimal medium (AAM) of Rudin et al.(1974) used contained the following ingredients per 1000 ml:

Salts:	KCl	3.0 g	Amino Acids:	L-arginine	125 mg
	NaCl	9.5 g		L-proline	200 mg
	$MgSO_4 \cdot 7H_2O$	1.3 g		L-glutamic acid	250 mg
	$(NH_4)_2SO_4$	4.0 g		L-valine	150 mg
	$CaCl_2 \cdot 2H_2O$*	22 mg		L-threonine	150 mg
	KH_2PO_4*	140 mg		L-phenylalanine	150 mg
	$FeSO_4 \cdot 7H_2O$**	6 mg		L-leucine	150 mg
	$MnSO_4 \cdot 4H_2O$**	10 mg		L-cystine*	80 mg
	Citric acid**	6 mg			
	Tris	12.1 g	Vitamins:	biotin*	0.1 mg
	Sodium citrate	5.0 g		thiamin*	2.0 mg
				nicotinic acid*	2.0 mg
Carbon source:				calcium pantothenate*	2.0 mg
	glucose*	5.0 g			

When a solid minimal medium was required, 15 gm agar (BBL) per 1000 ml was added and the pH adjusted to 7.9 with 1N HCl before autoclaving.

* = sterilized by filtration (0.45 μ, Millipore Filter Corp., Bedford, Massachusetts) and added separately to the autoclaved basal ingredients.

** = sterilized by filtration and added separately to the sterile basal ingredients as a solution containing 100 mg of $MnSO_4 \cdot 4H_2O$, 60 mg of $FeSO_4 \cdot 7H_2O$, and 60 mg of citric acid per 100 ml.

AAM Buffer (pH 7.9) had the following contents:

Na_2HPO_4 (anhydrous)	7.0 gm
KH_2PO_4	3.0 gm
NaCl	4.0 gm
$MgSO_4 \cdot 7H_2O$	0.2 gm
Water	to 1000 ml

Each salt was dissolved completely in the order given before adding the next. The buffer was sterilized in an autoclave for 15 minutes.

Incubation

Incubation temperature was 37 C unless otherwise specified. Incubation with shaking (90 cycles per minute, cpm) was performed in a water-bath (Forma Scientific Shaking Bath Model 2562-2040, Forma Scientific, Inc., Marietta, Ohio).

Mutants

Isolation of DNase-Deficient Mutants of *S. aureus* 7-8

The $DNase^-$ culture was obtained by the procedure of Omenn and Friedman (1970). *S. aureus* strain 7-8 was inoculated into 10 ml of TSB-YE-Ca, incubated overnight, and the cells harvested by centrifugation (Sorvall SS-3) at 5,000 x g for 10 minutes. The cells were washed once with 0.07 M sodium phosphate buffer (pH 7.0), and resuspended in 9 ml of the same buffer prior to treatment with the mutagen. A stock solution containing 4,000 µg of 1-methyl-3-nitro-1-nitrosoguanidine (NTG, Aldrich

Chemical Company, Milwaukee, Wisconsin) per ml water, sterilized by filtration, was added to the cell suspension to a final concentration of 800 µg/ml. All NTG solutions were prepared daily for use. The cell suspension was then incubated for 30 minutes. One-milliliter samples of the mutagen-treated cells were diluted in 50 ml of TSB and incubated overnight.

Three qualitative assays were performed for DNase activity: a) fluorescence on DNA-AO test medium, b) toluidine blue test, c) HCl test. Dilutions of the growth from the mutagen-treated culture were prepared in saline solution (0.85%, w/v, NaCl in H_2O) to give approximately 100 colonies per plate when streaked on DNA-acridine orange (AO) agar. This medium was prepared by adding 40 µg AO (Fisher Scientific Co.)/ml DNase Test Agar (BBL) before autoclaving. Plates of this medium had a brilliant yellow-green fluorescence. Release of nuclease from a $DNase^+$ colony was indicated by a dark halo of defluorescence when illuminated with UV light. Fluorescence persisted around $DNase^-$ colonies. In the toluidine blue test, dilutions of growth from the NTG-treated cultures were streaked onto DNase Test Agar plates and incubated overnight. Plates containing isolated colonies were flooded with a 0.1% (w/v) aqueous solution of Toluidine Blue O (Fisher Scientific Co.). A pink halo developed immediately around $DNase^+$ colonies while no such halo occurred with $DNase^-$ colonies. In the HCl test, colonies on DNase Test Agar plates were flooded with 1N hydrochloric acid. Any $DNase^+$ colonies were surrounded by a distinct clear zone, while $DNase^-$

colonies showed no clearing.

Presumptive DNase$^-$ colonies on DNA-AO agar were retested as above for stability of the character. A stable DNase$^-$ isolate was selected for later use and maintained on TSA-YE-Ca at 4C.

Isolation of Auxotrophs of S. aureus

The DNase$^-$ mutant of S. aureus was grown for 7 hours in 5 ml of TSB-YE in a 15-ml centrifuge tube, centrifuged (4500 x g) for 10 minutes (supernatant discarded), and the pellet resuspended in 5 ml of TSB-YE. A sterile solution containing 20 mg of NTG per ml water was added to the cell suspension to a final concentration of 200 µg/ml. The cells were incubated with NTG for 15 minutes in a water-bath, washed thrice with AAM buffer and once with TSB-YE. The washed cells were resuspended in 5 ml of TSB-YE. A 0.1-ml volume was transferred to 5 ml of fresh TSB-YE in a 15-ml centrifuge tube and incubated overnight. The growth from the mutagenized culture was diluted 1:10 in AAM buffer and 0.1 ml transferred to 10 ml of liquid AAM containing penicillin (7 µg/ml; potassium penicillin G; Pfizer Laboratories Division, Chas. Pfizer and Co., Inc.; New York, N. Y.). The culture was incubated with penicillin for 6 hours with shaking, diluted 1:10 and 1:100 in AAM buffer, and 0.1-ml amounts of the undiluted and diluted samples spread on TSA-YE plates. The plates were incubated overnight. Plates having 30-300 isolated colonies were replicated onto AAM agar plates by the method of Lederberg and Lederberg (1952) and the replica plates incubated overnight.

Presumptive mutants (no growth on AAM) were transferred from the TSA-YE plates to tubes containing 1 ml of AAM buffer for use as inocula for confirmation experiments. Each presumptive mutant was subsequently streaked onto TSA agar and AAM agar plates and incubated overnight to test whether they were mutants or replication failures. Cultures growing on both TSA and AAM agar plates were discarded. Cultures growing on TSA but not on AAM were streaked on eight AAM agar plates supplemented with amino-acids, vitamins, purines, and pyrimidines as indicated below:

Pool	1	2	3	4
5	adenine[a]	alanine[b]	ornithine[b]	glycine[b]
6	cytosine[a]	tryptophan[b]	aspartic acid[b]	isoleucine[b]
7	thymine[a]	tyrosine[b]	histidine[b]	methionine[b]
8	guanine[a]	lysine[b]	riboflavin[c]	uracil[a]

[a] 2 mg/ml

[b] 80 mg/ml of L form (160 mg/ml of DL form)

[c] 250 µg/ml

The designated concentration of each growth factor above was dissolved in separate 100-ml volumes of distilled water. The growth factors were then combined in equal volumes to form the pools shown in vertical columns 1 to 4, and in different combinations to form pools 5 to 8 as shown in the horizontal columns; sterilized by filtration; and stored at 4C. Each pool was added to liquid AAM or melted and cooled (50C) AAM agar (1 ml pool per 100 ml medium). Any single growth factor requirement showed as growth on one of the pools 1-4 and one of the pools 5-8 only.

The plates were incubated until colonies developed. The specific nutritional requirement of each suspected mutant was confirmed by streaking each (from the AAM buffer suspension) onto AAM agar plates supplemented with the single appropriate growth factor and incubated as above. Mutants having confirmed specific nutritional requirements were determined to be DNase$^-$ as before, transferred and maintained on TSA-YE-Ca slants at 4 C.

Reversion Rate to Prototrophy

The rate of reversion to prototrophy by auxotrophs selected for transductional and transformational analysis was determined as follows: The cells from two overnight TSA-slant cultures of a mutant were combined, washed 3 times in saline, and finally suspended in 2.0 ml saline. One-tenth milliliter portions were spread onto AAM agar plates and incubated 3-4 days before counting colonies that occurred. Total cell counts (colony forming units per ml, cfu/ml) were determined by preparing dilutions (1×10^{-8} and 1×10^{-9}) of the above auxotrophic-cell suspensions and streaking 0.1-ml volumes onto the surfaces of TSA-YE-Ca plates with a sterile glass applicator. Total colony counts were determined after overnight incubation. The reversion rate was described as the number of prototrophic colonies occurring on AAM agar plates per 10^{10} cfu on TSA-YE-Ca.

Syntrophism

Mutants having the same nutritional deficiency were tested for their ability to stimulate the growth of each other. The growth from an 18-24

hour TSA-slant culture of an auxotroph was suspended in 2.0 ml TSB-YE, transferred to a 15-ml centrifuge tube, centrifuged (4500 x g, 10 minutes, 4 C), washed once in saline, and resuspended in 2 ml of saline. Plates of AAM medium were prepared by spreading over their surfaces 0.1 ml of a dilute aqueous solution (0.8 µg/ml) of the particular amino acid or purine for which the mutant was deficient. Appropriate mutants were cross-streaked in all possible combinations on the prepared plates using sterile cotton swabs moistened in the cell suspensions. The plates were incubated until colonies appeared (4-5 days).

Mutants with the same phenotypic deficiency showing no evidence of syntrophism were selected for fine-structure analysis. These mutants were suspected to have defects within the same genetic locus.

Bacteriophages

Propagation of Bacteriophages

Growth from an 18-20 hour, TSA-slope culture of the appropriate Staphylococcus aureus propagating strain was suspended in 2 ml of TSB-YE-Ca. Two-tenths milliliter of the cell suspension, 0.1 ml sterile $CaCl_2$ solution (20,000 µg/ml), and 0.1 ml of the phage-stock suspension were added to 5 ml of melted and cooled (50 C) TSB containing 0.3% (w/v) agar. The contents were mixed and poured over the surface of a TSA-YE-Ca plate. Plates prepared in this manner were incubated in an upright position for 5 hours or until lysis was complete. The semi-solid layer was emulsified with a pipette in 5 ml of TSB-YE-Ca, transferred to a

15-ml centrifuge tube, and agar and cells sedimented by centrifugation (4500 x g, 10 minutes, 4 C). The phages were harvested by filtration (0.45 μ; Millipore Filter Corporation, Bedford, Massachusetts) of the fluid portion and stored in a sterile screw-capped tube at 4 C until used.

Titration of Phages

The number of phages per ml was determined as follows: One drop of an overnight TSB-YE-Ca culture of the appropriate S. aureus propagating strain was spread over the surface of a TSA-YE-Ca plate with a sterile glass spreader. Serial 10-fold dilutions of the phage harvest were prepared in sterile water, and 0.01 ml of each spotted on the cell lawn in appropriately numbered positions. The plate was incubated overnight. The titer, expressed as plaque-forming units/ml (pfu/ml), was determined by multiplying the number of plaques occurring with a particular dilution by the appropriate dilution factor.

Transduction

Sensitivity of DNase$^-$ Auxotrophs to Transducing Phages

Each double mutant was tested for sensitivity to viruses by suspending the growth from an overnight TSA-slope culture in 2 ml of TSB-YE-Ca. A sterile cotton swab moistened with this suspension was used to inoculate the entire surface of a TSA-YE-Ca plate. One drop of a 10^{-3} dilution of each phage stock (phages 29, 52, 53, 79, 80, 81, ∅11)

was placed on the prepared plates of each mutant. The plates were incubated overnight and examined for evidence of lysis in the test areas.

UV-Irradiation of Transducing Phages

All phage preparations used in transduction experiments had been exposed to UV irradiation. Phage preparations (5-10 ml) were placed in sterile glass petri dishes and, while being gently shaken, were irradiated for 60 seconds with a 15-watt germicidal lamp (General Electric) that delivered 0.65×10^4 ergs/cm^2-sec. to the surface of the liquid. All UV dosages used were measured with a Radiometer (model 65, Yellow Springs Instrument Company, Yellow Springs, Ohio). Phage preparations were titrated before and after UV exposure to measure the degree of killing.

Transductional Screening of Auxotrophs

The following screening test was employed to detect mutants useful for transductional analysis. The growth from an overnight TSA-slope culture of each auxotroph was suspended in 2.0 ml of TSB-YE-Ca. To 1.0 ml of this cell suspension was added 0.1 ml of a freshly-propagated, UV-irradiated phage preparation (grown on its propagating strain) of known titer (5.0×10^9 - 2.0×10^{10} pfu/ml), and the mixture incubated for 30 minutes with shaking. Following incubation, 1.0 ml of cold (4 C) citrate solution (0.5%, w/v, sodium citrate in water) was added to the tube and the contents mixed thoroughly. Cells were collected by centrifugation (4500 x g, 10 minutes, 4 C) resuspended in 2.0 ml of

TSB-YE-Cit, and incubated for 2.5 hours with shaking. Growth was collected by centrifugation as before, resuspended in 0.1 ml of saline and inoculated onto AAM agar plates with a sterile cotton swab. These plates were incubated for 4-5 days prior to counting the colonies. Controls consisted of cultures treated as above with 0.1 ml TSB substituted for phage suspension. This procedure was repeated exposing each lytic virus (grown on its propagating strain) to each of the mutants.

Transduction Procedure

In general, transduction was performed by mixing 2.0 ml (containing 1.0 - 5.0 x 10^{10} cfu/ml) of a cell suspension of an auxotroph (recipient) in TSB-YE-Ca with a known titer of UV-irradiated phages obtained after growth on a prototroph or auxotroph. The concentration of the phage preparation added was dictated by the multiplicity of infection (MOI, phage: cell) desired. The desired number of viruses were mixed with the cells and incubated for 30 minutes with shaking. Following incubation, an equal volume of cold (4 C) citrate solution was added and the suspension centrifuged (4500 x g, 10 minutes, 4 C). The cells were washed in 2 ml citrate solution and resuspended in 1.0 - 2.0 ml of citrate solution. Aliquots (0.1 ml) were spread over the surfaces of triplicate AAM agar plates. Counts of transductant colonies (prototrophs) were made after 3-4 days incubation. Controls were prepared using identical procedures except that an equal volume of TSB-YE-Ca was substituted for phages.

Total cell counts (cfu/ml) were determined by preparing dilutions (1×10^{-8} and 1×10^{-9}) of the transductant and control mixtures in sterile saline, and streaking 0.1-ml volumes onto the surfaces of TSA-YE-Ca plates with a sterile glass spreader. Total colony counts were determined after overnight incubation.

Reciprocal Transduction Procedure

Approximately $2 \times 10^{9} - 4 \times 10^{10}$ cells per ml of each of the auxotrophs were infected with phages (MOI = 1:2 - 1:8) grown on each of the mutants. Samples (0.1 ml) were spread on triplicate AAM agar plates for recovery of prototrophs. Since phage preparations differed to a substantial degree in their transducing "potentialities" for a constant number of bacteria (the number of transductions detected per given number of phage particles), the same phage preparation was used in the experimental combinations. The experiments with typing phage 80 were repeated two additional times using different virus and cell preparations.

Transformation

Isolation of Donor DNA

The DNA to be used for transformation was prepared according to Pattee and Neveln (1975) from an 18-hour culture of S. aureus strain 7-8 (prototroph). The donor cells from six 100-ml portions of TSB-YE were washed once (4000 x g, 20 minutes, 4 C) and suspended in 5 ml of 0.1 M Tris-hydrochloride buffer (pH 7.5) containing 0.15 M NaCl and

0.1 M ethylenediaminetetracetic acid (EDTA). Powdered Lysostaphin (Schwarz/Mann, Division of Becton-Dickinson and Company) was added to a final concentration of 10 units/ml and the suspension was incubated with shaking for 30 minutes. A 0.4-ml amount of sodium dodecyl sulfate (SDS, 5%, w/v, in 45% ethanol) was added and the mixture was shaken for an additional 30 minutes at room temperature. The resulting lysed suspension was mixed with an equal volume of steam-distilled phenol (saturated with 0.01 M Tris-hydrochloride buffer, pH 8.1), and the mixture was shaken for 30 minutes at room temperature. The resulting emulsion was broken by centrifugation (9000 x g) for 30 minutes at 4 C, and the upper aqueous phase was removed with a pipette. A second phenol extraction was performed. The aqueous phases were pooled and washed twice with ethyl ether. Excess ether was removed in a stream of N_2 gas. The DNA was precipitated by the addition of two volumes of cold (4 C) 100% ethanol, collected on a glass rod, and dissolved in 5.0 ml of 0.15 M NaCl plus 0.015 M sodium citrate. DNA preparations were stored at 4 C and used within 3 days.

A modified diphenylamine colorimetric reaction of Richards (1974) was used to determine the amount of DNA/ml. The diphenylamine reagent was prepared by dissolving 4.0 gm of steam-distilled diphenylamine in 100 ml of redistilled acetic acid containing 0.01% (v/v) paraldehyde. The reagent was stored in the dark and used within 1 month of preparation.

A standard curve was prepared for DNA analysis. A stock solution of 200 µg calf thymus DNA (Sigma, sodium salt, type V) per ml water was

prepared. Concentrations of 150, 100, 50, 25, 15, 10, 5, and 2 μg/ml were made in 1.5 N $HClO_4$ from the stock solution. Two-milliliter amounts of each of the above concentrations of DNA were pipetted into tubes containing 2 ml of diphenylamine reagent. Color was allowed to develop in the dark for exactly 24 hours at room temperature, and the absorbance (600 nm) was determined spectrophotometrically (Coleman 124, Hitachi). A standard curve (see Appendix) was determined by plotting the concentration of DNA (horizontal axis) against the corresponding absorbances (vertical axis). The concentration of an unknown DNA preparation was assayed as follows: 1.5 ml of 1.5 N $HClO_4$ was added to 0.5 ml of a donor DNA extract for acidification, 2 ml of the diphenylamine reagent was added, the mixture was placed in the dark for 24 hours at room temperature and the absorbancy determined as above. The amount of DNA per ml of donor preparation was determined from the standard curve previously described.

Preparation of Lysogenic Bacterial Recipients

Aspartic acid-deficient strains (Asp-$^{-}$4, Asp-$^{-}$7, Asp-$^{-}$11) were grown overnight in TSB-YE-Ca and 0.1-ml volumes spread over the surfaces of TSA-YE-Ca plates with a sterile glass spreader. About 0.2 ml undiluted phage Ø11 preparation was spotted onto the seeded area and incubated overnight. Any secondary growth occurring within the area of confluent lysis was inoculated into TSB-YE-Ca and cultures exposed to viruses as above until there was no evidence of lytic effect.

A strain was considered lysogenized when a) it was resistant to lytic infection by the lysogenizing phage, and b) UV-induction of a broth culture resulted in a lysate with a higher phage titer than controls. Failure of cultures of the recipients to show evidence of lytic infection when exposed to the lysogenizing phages on TSA-YE-Ca plates indicated immunity to this phage. Modification of the UV-irradiation technique of Gorrill and Gray (1956) was used for induction of bacteriophages in testing the recipients for lysogeny. Cultures to be induced were grown overnight in 10 ml of TSB-YE-Ca with shaking. Five-milliliter volumes of the culture were pipetted into two sterile glass petri dishes and the contents of one dish (experimental) were irradiated for 45 seconds with the 15-watt germicidal lamp while being gently shaken. The second (non-irradiated) sample served as a control. Cells from the irradiated and control cultures were harvested, resuspended in fresh TSB-YE-Ca, and incubated overnight in 15-ml centrifuge tubes. The cultures were then centrifuged (4400 x g, 10 minutes, 4 C) and the supernatants filtered as above. The phage titers of the supernatants were determined on S. aureus propagating strain 7-8.

The lysogenizing phage was indicated in parentheses following each auxotroph's strain number. For example, Asp-$^{-}$4 (Ø11) designated the aspartic acid-deficient mutant strain 4 lysogenized by phage Ø11.

Transformation Procedure

The recipients, S. aureus strains Asp-$^{-}$4 (Ø11), Asp-$^{-}$7 (Ø11), and Asp-$^{-}$11 (Ø11), were incubated for 15 hours in 5 ml TSB-YE-Cit. One-half

milliliter of each culture was then inoculated into 50 ml of TSB-YE-Cit in duplicate 300-ml erylemeyer flasks. All cultures were incubated with shaking until they reached an OD of 0.10-0.12 (approximately 1.5 hours). The OD was determined spectrophotometrically (Spectronic 20, Bausch and Lomb, 525 nm.). The cells were harvested (6000 x g, 20 minutes 4 C), washed once with 2 ml of 0.1 M Tris (hydroxymethyl) aminomethane-maleate buffer, pH 7.0, and suspended in 0.1 ml of the same buffer containing 0.1 M $CaCl_2$. The DNA preparation previously isolated was added to the experimental tubes to give a concentration of 6.9 µg/ml (near saturation). An equal amount of sodium citrate solution was added to a duplicate (control) tube instead of the DNA solution. The preparations were incubated for 30 minutes with shaking. Cells were collected by centrifugation (4500 x g, 10 minutes, 4 C), suspended in 1.0 ml of TSB-YE-Cit, and incubated for 30 minutes with shaking. The cells were again collected by centrifugation as above, suspended in 1.0 ml of sodium citrate solution and 0.1-ml aliquots spread over the surface of triplicate AAM agar plates. Counts of transformant colonies were made after 3-4 days incubation. Total cell counts (cfu/ml) were determined as previously described.

RESULTS

Nutritional Deficiency and Phage Sensitivity of Auxotrophs

The nutritionally-deficient (auxotrophic) mutants of the DNase$^-$ mutant of S. aureus strain 7-8 recovered have been listed in Table 1. Twenty-nine auxotrophic strains were selected. Nineteen strains were deficient for one of the amino acids: aspartic acid, histidine, glycine, lysine, or methionine. Ten strains required the addition of the purine guanine or the pyrimidine uracil for growth. Mutants having the same phenotypic deficiency listed in Table 1 failed to exhibit evidence of syntrophism (cross-feeding) when all possible combinations were cross-streaked on AAM medium.

The results of determination of sensitivity of auxotrophs to lysis by each of the seven possible transducing phages have been given in Table 2.

Transductional Screening

All phage strains used in transductional screening and in subsequent reciprocal-transduction experiments had been exposed to UV irradiation in attempts to enhance the rate of transduction. The effect of UV irradiation on the lytic titer of these phages has been shown in Table 3. Both an increase and decrease in lytic activity was observed.

Results from the transductional-screening analysis have been given in Table 4. Transductional screening was only attempted with those

Table 1. Auxotrophs of DNase⁻ *S. aureus* 7-8 obtained after chemical mutagenesis with NTG.

Type	Deficiency	Number auxotrophs	Mutant strain designation
Amino acid	Aspartic acid	11	Asp-⁻1 to Asp-⁻11
Amino acid	Histidine	1	His-⁻1
Amino acid	Glycine	1	Gly-⁻1
Amino acid	Lysine	2	Lys-⁻1, Lys-⁻2
Amino acid	Methionine	4	Met-⁻1 to Met-⁻4
Purine	Guanine	5	Gua-⁻1 to Gua-⁻5
Pyrimidine	Uracil	5	Ura-⁻1 to Ura-⁻5

Table 2. Sensitivity of auxotrophs to S. aureus transducing phages.

Auxotroph	Bacteriophages						
	Ø11	52	53	29	79	80	81
Control (7-8)	+	+	+	+	+	+	-
Asp-$^{-}$1	-	+	+	+	+	+	-
2	-	+	-	+	+	+	-
3	-	-	-	+	+	-	-
4	+	-	-	+	+	+	-
5	+	+	-	+	+	+	-
6	+	+	-	+	+	+	-
7	+	+	-	+	+	+	-
8	+	+	+	+	+	+	-
9	-	-	-	-	+	-	-
10	-	+	-	+	+	+	-
11	+	+	+	+	+	+	-
His-$^{-}$1	-	+	+	+	+	+	-
Gly-$^{-}$1	+	+	-	+	+	+	-
Lys-$^{-}$1	+	+	-	+	+	+	-
2	+	+	-	+	+	+	-
Met-$^{-}$1	-	+	+	+	+	+	-
2	-	-	-	+	+	+*	-
3	+	+	-	+	+	+	-
4	+	+	+	+	+	+	-
Gua-$^{-}$1	-	+	-	+	+	+	-
2	-	-	-	+*	+*	+*	-
3	-	+	-	+	+	+	-
4	-	+	-	+	+	+	-
5	-	-	+	+	+	+	-
Ura-$^{-}$1	+	+	-	+	+	+	-
2	-	-	-	-	+	-	-
3	-	+	-	+	+	+	-
4	+	+	-	+	+	+	-
5	-	-	-	+*	+*	+*	-

+ = lysis
- = no lysis
* = strains Gua-$^{-}$2 and Ura-$^{-}$5 showed reduced sensitivity (or greater resistance) to typing phages 29, 79, and 80 compared to the parent culture. Met-$^{-}$2 showed reduced sensitivity to typing phage 80

Table 3. Effect of UV-irradiation on transducing phages.

Bacteriophage	Bacteriophage titer[a] Before irradiation	After irradiation	Change in lytic activity
ø11	1.0×10^{10}	1.0×10^{10}	0
52	3.5×10^{9}	1.4×10^{9}	60% decrease
53	1.7×10^{6}	2.0×10^{7}	99.2% increase
29	8.0×10^{6}	6.0×10^{9}	99.9% increase
79	2.0×10^{10}	1.1×10^{10}	45% decrease
80	1.3×10^{11}	2.0×10^{10}	84.6% decrease

[a]values expressed as plaque-forming units per ml (pfu/ml)

Table 4. Transductional screening of auxotrophs.

Auxotroph	Control (reversion)	Bacteriophages					
		Ø11	52	53	29	79	80
Asp-⁻1	+/a		++/a	++/a	+++/a	+/a	++/a
2	++/a		-		-	-	++/a
3	+/a				+/a	++/a	
4	+/a	++/a			+/a	*/a	++/a
5	+++/a	*/a	++/a		++/a	+++/a	+++/a
6	+++/a	+++/a	++/*/a		++/*/a	+++/a	+++/a
7	++/a	+++/a	++/a		+/a	+++/a	+++/*/a
8	+/a	*/a	-	+/a	-	++/a	+/a
9	b					b	
10	+++/a		+++/a		++/a	++/a	+++/a
11	+/a	+++/a	+/a	+/a	++/a	+/a	++/a
His-⁻1	-		-	-	-	-	-
Gly-⁻1	b	b	b		b	b	b
Lys-⁻1	-	-	-		+++/a	-	-
2	-	-	-		+++/a	-	-
Met-⁻1	-		+/a	+/a	+++/a	++/a	-
2	-				+++/a	++/a	-
3	-	-	-		+++/a	-	-
4	-	-	-	+/a	+++/a	-	+/a
Gua-⁻1	-		++/a		+++/a	-	-
2	-				-	-	-
3	+/a		+/a		++/a	-	+/a
4	++/a		+/a		+/a	-	-
5	-			-	-	-	-
Ura-⁻1	+++/a	-	++/a		+++/b	+/a	+/a
2	+/a					-	
3	b		b		b	b	b
4	++/*	-	+/a		++/a	-	++/*/a
5	+++/a				+++/a	+++/a	+++/a

\+ = 10 or fewer colonies
++ = 10-100 colonies
+++ = more than 100 colonies
* = dense "minutes"

\- = no growth
a = clear background
b = confluent growth

culture-phage combinations shown in Table 2 to be lytically infective. Transductionally-competent culture-phage relationships can be identified from the results presented in Table 4. The controls revealed those cultures likely not able to be genetically analyzed because of high rate of spontaneous reversion to prototrophy, i.e., Asp-$^{-}$2, Asp-$^{-}$5, Asp-$^{-}$6, Asp-$^{-}$10, Gua-$^{-}$4, Ura-$^{-}$1, Ura-$^{-}$4, and Ura-$^{-}$5. Certain other culture-phage relationships showed evidence of abortive transduction, i.e., Asp-$^{-}$4 with typing phage 79, Asp-$^{-}$5 with phage Ø11, Asp-$^{-}$6 with typing phage 52 and 29, Asp-$^{-}$7 with typing phage 80, Asp-$^{-}$8 with phage Ø11 and Ura-$^{-}$4 with typing phage 80. Because the occurrence of abortive transduction with some phage-culture combinations implied transduction, but at a reduced and/or deficient capacity for recombination, these culture-phage relationships were less desirable as models for further genetic study.

Strains presented in Table 5 all showed some degree of transductional competency determined by comparison of the experimental (phage exposure) and control (spontaneous reversion) results of the transductional screening in Table 4. Transductants behaved prototrophically with no evidence of phage erosion (unstable lysogeny), or high reversion rate. Strain Asp-$^{-}$7 showed high transductional ability with typing phage 80 (Table 4) even though some abortive transductants were detected. Phage Ø11 was restricted to transduction of the aspartic-acid auxotrophs. Typing phage 29 showed highest transduction capabilities with the lysine-methionine groups of auxotrophs. The histidine, glycine and uracil auxotrophs showed no transductional capacity by any of the phages.

Table 5. Transductionally-competent culture-phage groups.

Auxotroph	Bacteriophages[a]					
	Ø11	52	53	29	79	80
Asp-⁻1		X	X	X		X
⁻3					X	
⁻4	X					X
⁻7	X				X	X
⁻8					X	
⁻11	X			X		X
Lys-⁻1				X		
⁻2				X		
Met-⁻1		X	X	X	X	
⁻2				X	X	
⁻3				X		
⁻4			X	X		X
Gua-⁻1		X		X		
⁻3				X		

[a]X = transducible by the bacteriophages

Fine-structure Transductional Analysis

Auxotroph strains Asp-$^{-}$4, Asp-$^{-}$7, and Asp-$^{-}$11 were selected for fine-structure analysis using typing phage 80 and phage Ø11. These three mutants had the same phenotypic deficiency, showed no syntrophism, and consequently were suspected to have defects within the same genetic locus. A number of other transducible systems for genetic study were also indicated in Table 5. Examples are; Asp-$^{-}$1, and -$^{-}$11 by typing phage 29; Asp-$^{-}$3, -$^{-}$7, and -$^{-}$8 by typing phage 79; Asp-$^{-}$1, -$^{-}$4, and -$^{-}$11 by typing phage 80; Met-$^{-}$1, -$^{-}$2, -$^{-}$3, and -$^{-}$4 by typing phage 29; Gua-$^{-}$1 and -$^{-}$3 by typing phage 29.

Effect of the Multiplicity of Infection on the Transductional Frequency

Preliminary experimentation with typing phage 80 and phage Ø11 showed that the multiplicity of infection used in the transductional suspension significantly influenced the transductional frequency. Accordingly, all experiments were made at the multiplicities yielding maximum and reproducible transductional frequencies. Typing phage 80 was more virulent than phage Ø11 when grown on the aspartic-acid mutants, and therefore yielded significantly higher lytic titers (5×10^{10} - 1×10^{11} pfu/ml). The typing phage 80 preparations were diluted to produce MOI's of 1:2 to 1:8. Phage Ø11, being less virulent than typing phage 80, had to be passed through the host culture several times before reaching the highest lytic titer (1×10^{9} - 1×10^{10} pfu/ml). These phage preparations

were used undiluted, making MOI's of 1.5:1 to 1:1.3. Cell titers used in all transduction experiments were 8×10^9 - 5×10^{10} cfu/ml.

The rates of reversion of the three aspartic-acid mutants to prototrophy have been presented in Table 6. Marked variation in the sizes of the revertant colonies on AAM agar was noted. In comparing the transductional frequencies from the screening procedure (Table 4) with the reversion rates (Table 6) our results indicated transduction frequency with the three aspartic-acid mutants was expected to be 10^3 to 10^4 times greater than the reversion rates.

Recipient Capacity

The three mutant strains (Asp-$^-$4, Asp-$^-$7, Asp-$^-$11) were infected with typing phage 80 grown on parent strain 7-8 (80/7-8) to determine the recipient capacity (efficiency to produce prototrophic transductants). The experiment was made with a single lysate of phage 80/7-8 so that the titer and phage preparation were identical for each. The results have been presented in Table 7.

Reciprocal Transductions Between Aspartic-acid Mutants

In Table 8, results have been presented of three experiments in which cultures of each of the aspartic acid mutants ("Auxotroph" column) were infected with typing phage 80 grown on each of these mutants ("Transducing virus" column). Representative transductant clones (≈25 per experiment) were sub-cultured and tested for lytic sensitivity to

Table 6. Reversion rate to prototrophy by aspartic-acid auxotrophs.

Auxotroph	Reversion rate ($x10^{-10}$)[a]
Asp-⁻4	16.20
Asp-⁻7	8.76
Asp-⁻11	2.55

values presented were averages of colony counts from 7 plates

[a]rate determined by dividing the average revertant colony count by the total cell count

Table 7. Recipient capacity of aspartic-acid mutants of S. aureus.

Auxotroph	Recipient capacity[a]
Asp-$^{-}$4	2398
Asp-$^{-}$7	700
Asp-$^{-}$11	2930

[a]recipient capacity expressed as number of transductants per 10^{10} phage particles (corrected for reversion)

Table 8. Number of prototrophic colonies obtained upon reciprocal transductions between aspartic-acid-auxotrophs using typing phage 80.

Auxotroph	Transducing virus	Number of transductants Experiments[a] 1	2	3	Average number of transductants[b]
Asp-⁻4	80/4	0	0	0	0
Asp-⁻4	80/7	6900	7	450	2452
Asp-⁻4	80/11	200	7	348	185
Asp-⁻7	80/4	540	28	49	206
Asp-⁻7	80/7	0	0	0	0
Asp-⁻7	80/11	545	96	129	257
Asp-⁻11	80/4	548	28	71	216
Asp-⁻11	80/7	8000	222	80	2767
Asp-⁻11	80/11	0	0	0	0

[a]values corrected for reversion

[b]average (3 experiments) frequency of transduction expressed as number of transductants per 10^{10} phage particles

typing phage 80. All clones tested were found to be sensitive to typing phage 80 and were assumed nonlysogenic for this phage.

Reciprocal transduction of the three aspartic-acid auxotrophs with phage Ø11 was attempted to confirm the results obtained in transductions with typing phage 80. The results have been presented in Table 9. Frequencies of transductions using phage Ø11 were low and reproducibility was difficult. We have presented the results of only a single experiment giving the highest rates obtained.

Lysogenic-auxotrophic strains Asp-$^{-}$4(Ø11), Asp-$^{-}$7(Ø11), and Asp-$^{-}$11(Ø11) were no longer sensitive to lytic infection by typing phage 80; however, a separate reciprocal transduction experiment using these strains and typing phage 80 was attempted. The numbers of revertants obtained closely approximated the numbers of prototrophic colonies recovered from the combinations of phages and auxotrophs used in the transduction attempts. Therefore, no or insignificant transduction occurred with typing phage 80 and the three phage Ø11 lysogenic aspartic-acid auxotrophs.

Transformation

Donor DNA obtained from parent strain 7-8 was used in transformation experiments with each of the lysogenic aspartic-acid auxotrophs[Asp-$^{-}$4(Ø11) Asp-$^{-}$7(Ø11), and Asp-$^{-}$11(Ø11)] as recipients. The results of a single experiment have been presented in Table 10.

Table 9. Reciprocal transductions between aspartic-acid-auxotrophs using phage Ø11.

Auxotroph	Transducing virus	Transductants[a]
Asp-⁻4	Ø11/4	0
Asp-⁻4	Ø11/7	100
Asp-⁻4	Ø11/11	3
Asp-⁻7	Ø11/4	111
Asp-⁻7	Ø11/7	0
Asp-⁻7	Ø11/11	11
Asp-⁻11	Ø11/4	111
Asp-⁻11	Ø11/7	130
Asp-⁻11	Ø11/11	0

[a]frequency of transduction expressed as number of transductants per 10^{10} phages particles (corrected for reversion)

Table 10. Number of prototrophs obtained from transformation of phage Ø11 lysogenic auxotrophs with DNA from parent strain 7-8

Recipient	Number of recipients (cfu/ml)	Number of Asp^+ transformants/ml		Frequency of transformation[a]
		+DNA	-DNA	
Asp-⁻4(Ø11)	7.5×10^8	1.05×10^3	0	1.4×10^{-4}%
Asp-⁻7(Ø11)	1.2×10^{10}	2.08×10^4	0	1.7×10^{-4}%
Asp-⁻11(Ø11)	3.2×10^9	5.20×10^4	0	1.6×10^{-3}%

[a]values expressed as number of transformants per number of recipients used in the original inoculum x 100

DISCUSSION

Selection of Phages and Bacterial Mutants for Use in Genetic Analysis

Of the twenty-nine auxotrophic strains that were selected following mutagenic treatment of the S. aureus parent strain 7-8, thirteen showed no evidence of reversion (Tables 1 and 4), and could have been deletion or multisite mutations. However, further genetic analysis would be required to confirm this premise. The other sixteen auxotrophs did exhibit reversion to prototrophy, and were probably point mutations. The high proportion of mutants showing no reversion was expected considering the mutagenic agent used in this study. The mode of action of NTG was described by Cerda-Olmedo and Hanawalt (1967, 1968) as producing mostly base-pair substitutions and some deletion mutations. NTG has been shown to be a potent mutagen and was expected to produce a large number of multisite mutations that would not revert to prototrophy.

Since cell wall construction and cellular sensitivity to lysis by a specific phage have been shown to be genetically controlled, changes in phage-sensitivity patterns of the auxotrophs were expected following mutagenesis of the parent strain with NTG. The data in Table 2 showed that every auxotrophic strain except Asp-$^{-}$8, Asp-$^{-}$11, and Met-$^{-}$4 gained resistance to one or more of the phages used in this investigation. The parent strain 7-8 was insensitive to typing phage 81. Lack of sensitivity to lytic infection by this phage served as a control to assist in detection

of any contaminants. Dowell and Rosenblum (1962a) reported that staphylococci immune to a particular phage were able to be transduced by it, however, at a markedly reduced rate. Thus, only our auxotrophs retaining lytic sensitivity to the staphylophages were considered for transductional analysis.

The transductional screening (Table 4) and the subsequent determination of the transductionally-competent culture-phage groups (Table 5) expedited the selection of systems for fine-structure analysis. All of the staphylophages used in this investigation showed competency in transducing some of the auxotrophs. It was unnecessary to determine actual rates of reversion and transduction in the screening tests. Evidence of abortive transduction was revealed with some phage-culture combinations. Abortive transduction may represent a deficient capacity for recombination, therefore, only one auxotroph-phage combination, Asp-$^{-}$7/phage 80, that showed evidence of abortive transduction but also a high rate of recombination was used for further fine-structure analysis.

Different dosages of UV irradiation would have been required to produce the same degree of inactivation for typing phages 52, 79, and 80 (Table 3). This difference in rate of inactivation was probably due to differences in phage titer exposed and procedure. The difference in rate of phage inactivation by the UV treatment may have affected the transductional-screening results (Table 4).

Typing phages 29 and 53 showed approximately equal increase in lytic activity following UV exposure (Table 3) however, typing phage 53-auxotroph combinations did not show nearly the transductional activity as

typing phage 29-auxotroph combinations (Table 4). Since this effect was observed for a particular phage strain exposed to a number of auxotrophs the basis of the effect probably resided in the phages, not the cells. Thus, although virulence and transductional capacity were probably unrelated for typing phage 29 they were possibly related in typing phage 53. A similar relationship may have existed for phage Ø11 which showed no change in lytic titer following exposure to UV dosage used (Table 3) and showed lower frequencies of transduction (3.3×10^{-10} to 7.7×10^{-8}) of the aspartic-acid auxotrophs (Table 9), when compared to the transductional frequencies (5.4×10^{-8} to 3.6×10^{-7}) by typing phage 80 (Table 8) which was 84.6% inactivated upon exposure to UV-irradiation (Table 3). Transductional experiments using UV-irradiated and non-irradiated preparations of these phages should be performed to verify this hypothesis. No further studies were done to elucidate the relationship between UV-irradiation, transductional capacity and lytic activity of the phages since transductionally-competent systems had been defined for typing phage 80 and phage Ø11.

Fine-Structure Genetic Analysis

Aspartic-acid auxotrophs were chosen for fine-structure analysis partly because aspartic-acid synthesis probably involved a single enzyme promoting transfer of an amino group from glutamic acid to oxaloacetic acid yielding aspartic acid. This premise was, in part, supported by

negative syntrophic results with all 11 of the aspartic-acid mutants investigated. The molecular weight of aspartate aminotransferase for S. aureus was unknown, however, the same enzyme in Bacillus subtilis had molecular weight of approximately 53,000 (The Enzymes, Vol. II by P. D. Boyer, article by Snell and DiMari, 1970). Assuming the molecular weight of the average amino acid was 120 there would be approximately 442 amino-acid residues in this enzyme. Since 3 nucleotides, each having an average molecular weight of 400, were required to code for each amino acid, a piece of DNA with a molecular weight of 5.3×10^5 would be required to contain nucleotide information for the enzyme aspartate aminotransferase. The mean molecular weight of DNA of transducing fragments carried by staphylophages was not known, however, typing phage 80 DNA had a molecular weight of 3.1×10^7 (Pariza and Iandolo, 1974). Because genome size appeared to be related to the size of the phage size, this fragment of DNA would be about 60 times larger than the amount of DNA proposed to code for aspartate aminotransferase.

The auxotrophs, Asp-$^-$4, Asp-$^-$7, and Asp-$^-$11, were selected for fine-structure analysis using typing phage 80 and phage Ø11 since they were transducible by these phages (Table 4). The three mutants had the same phenotypic deficiency, showed no syntrophism, and consequently were suspected to have defects within the same chromosomal genetic locus.

The difference in the spontaneous reversion rates of the three auxotrophs, Asp-$^-$4, and Asp-$^-$7, and Asp-$^-$11, (Table 6) showed a variation

in stability of the mutation. The larger prototrophic colonies observed represented true reverse mutations, while the smaller colonies probably reflected mutations of a suppressor gene. A similar situation was observed in studies of purine-dependent mutants of Salmonella typhimurium (Yura, 1956) and with tryptophan-dependent mutants of S. aureus (Ritz and Baldwin, 1962).

The multiplicity of infection (MOI) used in the screening experiments significantly influenced the transductional frequency. An inverse relationship was observed between MOI used and rate of transduction. Kloos and Pattee (1965) reported similar results in transductional studies of histidine-dependent mutants of S. aureus using typing phage 83. A low MOI (host cells in excess of phage particles) was found to give maximum and reproducible frequencies of transduction in their investigation.

Sodium citrate (0.5% w/v) was added to the transductional mixture to enhance recovery of transductants by preventing lysis by the free phages. Sodium citrate served to bind the calcium present. A lack of calcium has been shown to inhibit the adsorption of group B phages to staphylococcal cells (Rountree, 1951).

The establishment of lysogeny was not required for staphylococcal transduction using typing phage 80, a non-lysogenizing variant of typing phage 52A (Rountree, 1959; Comtois, 1960). Typing phage 80 had been previously shown to be capable of transduction of the tryptophan loci (Ritz and Baldwin, 1961) of novobiocin-resistance (Dowell and Rosenblum, 1962a)

and other markers (Pattee and Baldwin, 1961; Dowell and Rosenblum, 1962b). Our results with typing phage 80 supported this hypothesis. The phage 80-mediated aspartic-acid transductants were shown to be non-lysogenic in our experiment using low MOI's. We did not test the phage Ø11 transductants for lysogeny.

Theoretically, the number of wild-type recombinants recovered on minimal medium from reciprocal-transduction experiments indicated the distance between the alleles involved, those further apart producing relatively more recombinants. Only a single order of the alleles was expected from data of reciprocal-transduction experiments. The position of one particular mutant marker in relation to two other mutant sites has been determined by adding the numbers of prototrophs recovered from reciprocal-transduction experiments between pairs of mutants and determining the best "fit". From the data presented in Table 8, it was possible to construct a linkage map of the order and relative distances between the three aspartic-acid mutation sites based upon the numbers of prototrophic recombinants obtained in reciprocal-transduction experiments using typing phage 80 (Fig. 1). The relative distances (in percentages) between the mutant sites were determined by dividing the smaller number of recombinants by the larger number of recombinants for each pair of reciprocal crosses and multiplying the quotient by 100.

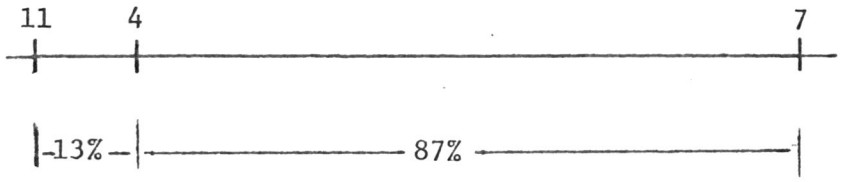


Fig. 1. The order of mutant sites (Asp-$^{-}$4, Asp-$^{-}$7, Asp-$^{-}$11) on the chromosome of S. aureus 7-8 as determined by transductional analysis with typing phage 80.

In order to confirm the above linkage map, a map was constructed (Fig. 2) using the data obtained from reciprocal transductions among the three aspartic-acid auxotrophs using phage Ø11 (Table 9).

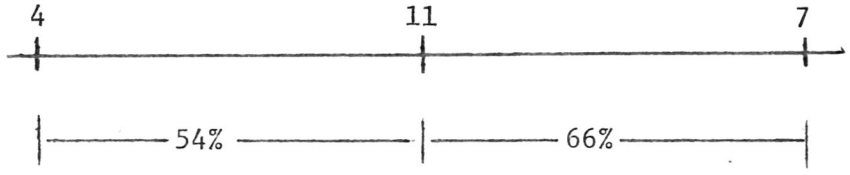


Fig. 2. The order of mutant sites (Asp-$^{-}$4, Asp-$^{-}$7, Asp-$^{-}$11) on the chromosome of S. aureus 7-8 as determined by transductional analysis with phage Ø11.

Both the relative order and distances of the mutant sites differed in these two maps. Because the experiments with phage Ø11 were only done once while the experiments with typing phage 80 were done three times the map presented in Figure 1 was considered more reliable than the map presented in Figure 2. Transductional experiments using phage Ø11 must be repeated before comparison of these results with the results of the experiments using typing phage 80 would be justifiable.

Reciprocal-transduction experiments with the phage Ø11-lysogenized aspartic-acid mutants and typing phage 80 were only attempted at the same

MOI's used in the transductional experiments using non-lysogenized auxotrophs. Use of higher MOI's could be explored to determine if increased rates of transduction could be effected with this system.

Hartman (1956) reported that the determination of the order of non-identical alleles by reciprocal transduction was only an approximation, since the method of transductional analysis was not precise enough for definitive placement of extremely closely linked markers. In order to verify the map presented in Figure 1, a map was prepared by determining the recipient capacity of these aspartic-acid mutants according to the method of Kloos and Pattee (1965). These workers proposed the donor fragments which participated in transduction of histidine-requiring auxotrophs to prototrophs by typing phage 83 grown on histidine-independent donor strain (655) had been preferentially broken at a specific point yielding homogeneous transducing particles. Pattee et al. (1968) reported that the point of termination of the histidine-transducing fragment, and hence one breakage point of the donor chromosome, was a predetermined characteristic of the S. aureus chromosome which was not influenced appreciably by the transducing phage. The linkage map presented in Figure 3 was constructed from the data in Table 7 and represented the ability of the mutants to form aspartic-acid-independent transductants when infected with typing phage 80 cultivated on the parent strain 7-8.

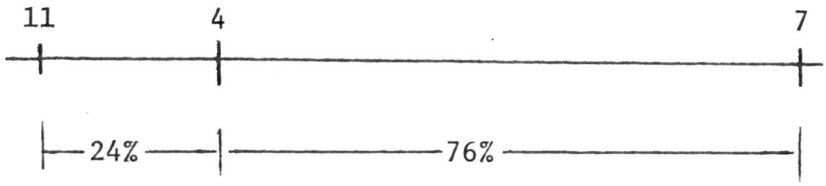


Fig. 3. The order of mutant sites (Asp-$^{-}$4, Asp-$^{-}$7, Asp-$^{-}$11) on the chromosome of S. aureus 7-8 as determined by recipient capacity.

According to this hypothesis, the chromosome of S. aureus 7-8 could have been fragmented somewhere to the "right" of the Asp-$^{-}$7 site during formation of the donor fragments. Thus, the frequency of transduction of a mutant site by a piece of genome from the parent strain was determined by the position of that mutation with respect to the terminus of the donor fragment. The mutant Asp-$^{-}$11, whose wild-type allele was located the greatest distance (relative to Asp-$^{-}$4 and Asp-$^{-}$7) from the hypothetical fragment terminus, provided the greatest opportunity for recombination to occur; hence, Asp-$^{-}$11 demonstrated the highest transductional frequency. The mutants, Asp-$^{-}$4 and Asp-$^{-}$7, being relatively closer to the terminus, exhibited relatively fewer transductants as compared to Asp-$^{-}$11. This map was in agreement with the map obtained from results of reciprocal transductions with typing phage 80 (Fig. 1).

Ordering of mutation-sites by comparison of the relative recipient capacities of the aspartic-acid-dependent mutants would probably be more reliable than by determining their relative rates of reciprocal transduction. The transducing fragments in the latter were probably more heterogeneous because they were prepared from the three "different" chromosomes of the

aspartic-acid mutants. Transducing fragments involved in recipient-capacity experiments were more homogeneous both because of the proposed preferential breaking point and because the fragments were prepared from a single chromosomal preparation.

In transformation, the size of the transforming segments was predetermined by the degree to which the chromosomal DNA was fragmented during extraction from donor bacteria and subsequent purification. Consequently, transforming DNA fragments varied within and between preparations. This possible variation in genetic content of the donor DNA fragments must be considered when mapping the three aspartic-acid mutation sites. The mean molecular weight of transforming fragments of staphylococcal DNA were not known, however the mean molecular weight of transforming preparations of pneumococcal DNA was 5×10^6 (Fluke et al. 1952). The molecular weight of a DNA fragment containing the necessary nucleotides coding for aspartate aminotransferase was previously proposed to be about 5.3×10^5. Most fragments of DNA containing any genetic information for synthesis of this enzyme would therefore have carried the total information and would be able to form prototrophic recombinants with each of the three aspartic-acid auxotrophs.

A hypothetical map of the order of the mutation sites can be constructed assuming that on the average the transforming donor fragments are "homogeneous" in that they carry all three of the wild-type alleles corresponding to the three aspartic-acid mutation sites. Excluding multi-recombinational events,

the frequency of transformation of a mutant would be directly related to the position of the nucleotide sequence correcting the defect on the donor chromosomal fragment relative to the end of that fragment. The site furthest from the terminus had the greatest opportunity for cross-over between it and the terminus and thus the highest transformational frequency (Table 10). The "terminus" of the donor fragment was assigned somewhere to the "left" of the allelic site Asp-⁻4 as illustrated in Figure 4. Mutations Asp-⁻4, Asp-⁻7, and Asp-⁻11 were positioned in relation to the terminus by their recombinational (transformational) frequencies.

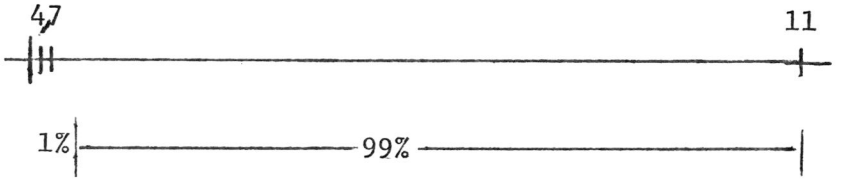


Fig. 4. The order of mutant sites (Asp-⁻4, Asp-⁻7, Asp-⁻11) on the chromosome of S. aureus 7-8 as determined by transformational analysis.

This map does not agree with the maps obtained by transductional analyses (Figs. 1, 2, and 3). Repeated determinations of the transformational frequencies of the three aspartic-acid mutation sites should be performed before valid comparisons can be made between the maps obtained from transductional and transformational analyses.

SUMMARY

Twenty-nine DNase$^-$ auxotrophic strains obtained after mutagenic treatment of the Staphylococcus aureus parent strain 7-8 with N-methyl-N'-nitro-N-nitrosoguanidine were selected for use in fine-structure genetic analysis. The auxotrophs were divided into two groups, those that showed evidence of reversion, probably representing point mutations, and those that showed no evidence of reversion, probably representing deletion or multisite mutations. Auxotrophs having point mutations were proposed to have chromosomal defects within the same genetic locus as determined by the lack of syntrophism.

The auxotrophs having point mutations were tested for lytic sensitivity to the serological group B staphylococcal typing phages 52, 53, 29, 79, 80, 81 and phage Ø11. Auxotrophs and their respective lytic phages were designated for possible use in transductional studies. A transductional screening test was used to identify transductionally competent phage-auxotroph groups for use in fine-structure genetic analysis. Each of the staphylophages showed competency in transducing some portion of the auxotrophs.

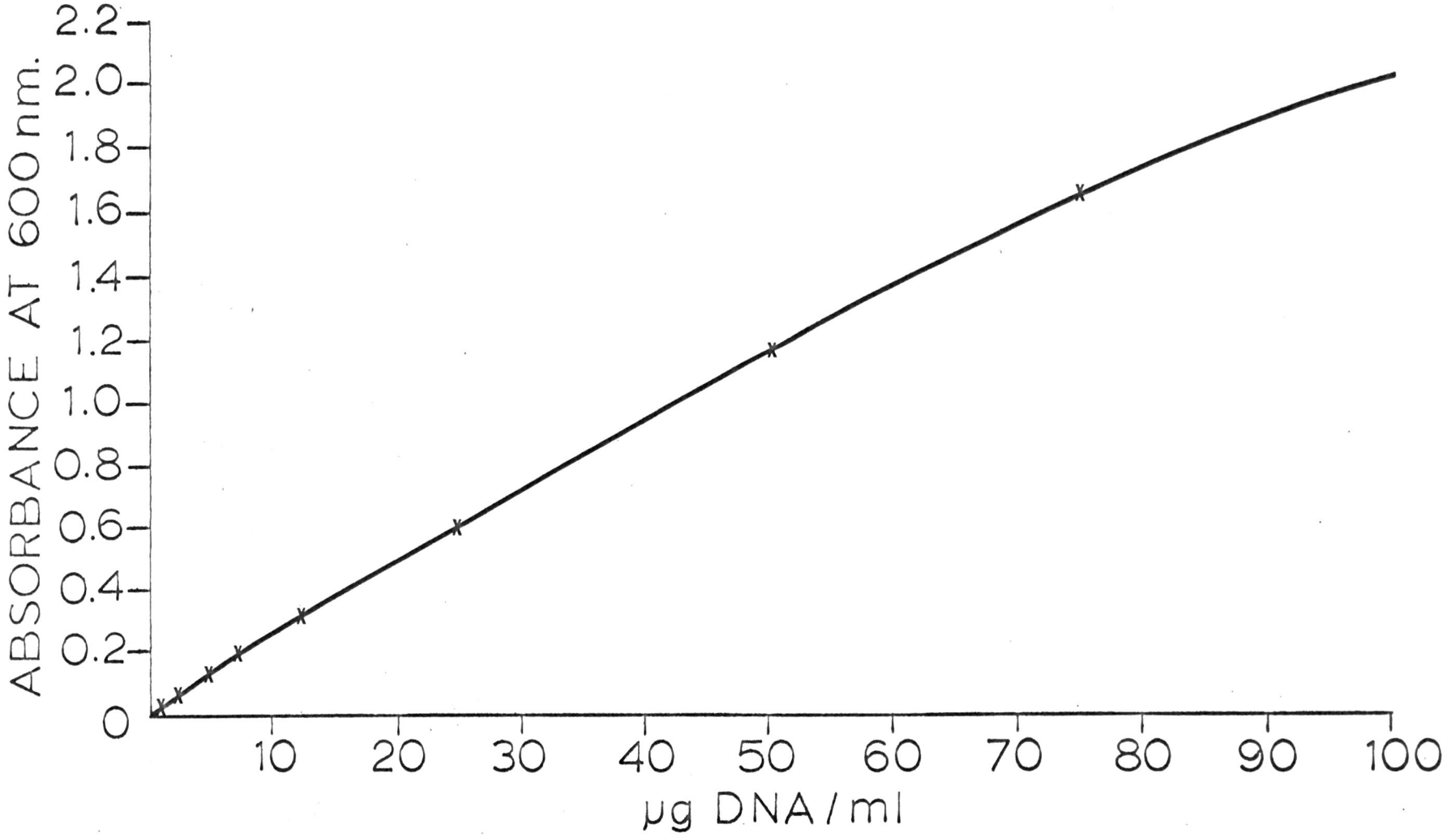
The aspartic-acid-deficient auxotrophs, Asp-$^-$4, Asp-$^-$7, and Asp-$^-$11, were selected for transductional analysis using typing phage 80 and phage Ø11 and for transformational analysis. Lysogeny was shown not to be a requisite for the aspartic-acid transductions. Transductional frequencies

of the phage Ø11-lysogenized mutants [Asp-$^{-}$4(Ø11), Asp-$^{-}$7(Ø11), and Asp-$^{-}$11 (Ø11)] using typing phage 80, were lower than the transductional frequencies using the non-lysogenized auxotrophs and the same phage. Phage Ø11 was not as effective a transducing phage for the non-lysogenized auxotrophs as typing phage 80.

Intragenic maps of three aspartic-acid mutation sites on the chromosome of S. aureus were prepared from data obtained by three methods; reciprocal-transduction analysis using typing phage 80 and phage Ø11, recipient-capacity determination using typing phage 80, and transformational analysis of the phage Ø11-lysogenized mutants. There was agreement in the order and relative position of the three aspartic-acid mutation sites determined from results of reciprocal-transduction experiments using typing phage 80 and recipient-capacity experiments. Results of preliminary experiments for both transductional analysis with phage Ø11 and transformational analysis of these mutants were inconclusive.

APPENDIX

Standard curve for DNA analysis


ABSORBANCE AT 600 nm.
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
80
90
100
µg DNA / ml

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