Role of bacteriophage in the acquisition of competence for genetic transformation and transfection in Staphylococcus aureus and related studies

Nancy Elizabeth Thompson
Iowa State University
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ROLE OF BACTERIOPHAGE IN THE ACQUISITION OF
COMPETENCE FOR GENETIC TRANSFORMATION AND
TRANSFECTION IN STAPHYLOCOCCUS AUREUS AND
RELATED STUDIES.

IOWA STATE UNIVERSITY, PH.D., 1979
Role of bacteriophage in the acquisition of competence for genetic transformation and transfection in *Staphylococcus aureus* and related studies

by

Nancy Elizabeth Thompson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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In Charge of Major Work

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For the Major Department

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Iowa State University
Ames, Iowa

1979
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>History and Incidence of Transformation and Transfection</td>
<td>5</td>
</tr>
<tr>
<td>Genetic Control of Competence and Transformation</td>
<td>16</td>
</tr>
<tr>
<td>Natural Competence</td>
<td>18</td>
</tr>
<tr>
<td>Expression of competence</td>
<td>18</td>
</tr>
<tr>
<td>Binding and uptake of DNA</td>
<td>29</td>
</tr>
<tr>
<td>Ca$^{2+}$-Induced Competence</td>
<td>35</td>
</tr>
<tr>
<td>Expression of competence</td>
<td>35</td>
</tr>
<tr>
<td>Binding and uptake of DNA</td>
<td>36</td>
</tr>
<tr>
<td>Competence in <em>Staphylococcus aureus</em></td>
<td>39</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>47</td>
</tr>
<tr>
<td>Bacterial Strains and Bacteriophages</td>
<td>47</td>
</tr>
<tr>
<td>Culture Media</td>
<td>52</td>
</tr>
<tr>
<td>Reagents and Buffers</td>
<td>53</td>
</tr>
<tr>
<td>Propagation of Bacteriophages</td>
<td>54</td>
</tr>
<tr>
<td>Purification of Bacteriophages</td>
<td>55</td>
</tr>
<tr>
<td>Isolation of Deoxyribonucleic Acid (DNA)</td>
<td>56</td>
</tr>
<tr>
<td>Bacterial DNA</td>
<td>56</td>
</tr>
<tr>
<td>Bacteriophage DNA</td>
<td>57</td>
</tr>
<tr>
<td>Transformation Procedure</td>
<td>58</td>
</tr>
<tr>
<td>Transfection Procedure</td>
<td>59</td>
</tr>
<tr>
<td>Transduction Procedure</td>
<td>60</td>
</tr>
</tbody>
</table>
Mutagenesis Procedure

Sera

Preparation of antisera

 Normal rabbit serum

 Precipitation of gamma-globulins

 Antiserum neutralization test

Bacteriophage Typing

Sonication of Cells

RESULTS

Preliminary Observations

Transfer of Competence with Growth Media

 Comparison of growth media from 8325nov-142 and 8325-4(ø11)

 Free phage content of media

 Neutralization of the competence-conferring activity of the media

 Assay for lysogeny

 Kinetics of the acquisition of competence

Incidence of Phage-conferred Competence

 Survey of phages for the ability to confer competence to 8325-4

 Survey of representative strains for the ability to demonstrate competence after exposure to phage

 Assay for congression

 Optimum conditions for phage-conferred competence
Multiplicity of infection 91
Phase of growth 93
Rate of Growth 93
Exposure time to phage 98
Inhibition of Competence 103
Effect of φ11-antiserum 104
Effect of sodium dodecyl sulfate (SDS) 108
Effect of Pronase 111
Detection of Competence-Conferring Components in Phage Lysates 120
Ultraviolet (UV) irradiation of phage lysates 121
Competence-conferring ability of purified phage 124
Effect of Pronase on the competence-conferring activity of the 80α supernatant 127
Attempts to dissociate phage particles 129
Effect of sonication 129
Effect of repeated freezing and thawing 132
Effect of dimethyl sulfoxide 132
Attempts to identify a mutant with increased competence-conferring activity 134
Suppressor-sensitive mutants 134
Temperature-sensitive mutants 135
Separation of pfu from competence-conferring components in 80α lysates 137
Ammonium sulfate precipitation 138
Precipitation with polyethylene glycol 138
Ultrafiltration 141
Isopycnic CsCl gradients 142
Ficoll gradients 145
Effect of Normal Serum on the Enhancement of Transfection Frequencies 146
Survey of sera 149
Serum concentration 151
Duration of enhancement 151
Attempts to identify the serum component responsible for enhancement 146
Inactivation of complement 156
\((\text{NH}_4)_2\text{SO}_4\) precipitation 156
Pronase treatment 156
Analysis of commercially prepared serum fractions 157
DISCUSSION 159
SUMMARY 176
BIBLIOGRAPHY 180
ACKNOWLEDGEMENTS 204
INTRODUCTION

The transfer of deoxyribonucleic acid (DNA) between bacterial species occurs by three basic modes: conjugation, transduction, and transformation. All three modes have been used extensively to characterize the genetic complement of several bacterial species and to investigate many fundamental problems of molecular genetics. However, very little is known of the actual mechanism(s) involved with the transfer of DNA molecules across bacterial membranes and walls.

Of the three modes of genetic exchange in bacteria, transformation is unique in not requiring a protein vector to transport DNA from donor to recipient. Therefore, transformation is experimentally the most versatile mode, allowing in vitro manipulation of the DNA.

Genetic transformation in bacteria is an alteration in phenotype resulting from the uptake of exogenously supplied DNA. Transformation is a multi-step process that involves adsorption of the DNA to the cell surface, transport of the molecule into the cell, recombination of the donor DNA with the recipient genome (or establishment of the donor DNA as a self-replicating entity within the recipient cell), and the expression of the newly acquired genetic material. Obviously, each of these events might involve several complex and integrated processes. Numerous investigators have attempted to characterize these processes in the various bacterial species in which a transformation system has been described. However, despite the vast investigative effort, most of the physiology of transformation remains an enigma.
Closely related to genetic transformation is the process of transfection. Transfection is the infection of cells by isolated viral nucleic acid which generally results in a lytic infection (Földes and Trautner, 1964). While the fate of the acquired DNA is generally different, transfection resembles transformation closely with respect to the adsorption and penetration of DNA.

Generally speaking, not every cell of a population can undergo transformation. In addition, transformation can be demonstrated only under specific environmental and cultural conditions. Those cells which are capable of undergoing transformation or transfection when exposed to genetically active DNA are referred to as "competent" (Thomas, 1955). Acquisition of the physiological state of competence appears to be requisite to the irreversible binding and transport of DNA in all transformable systems so far described.

The primary objective of this study was to investigate some of the parameters involved with the acquisition of competence in Staphylococcus aureus. The transformation system of this organism is of particular interest because of its utility in examining the genomic structure of this important opportunistic pathogen (Lindberg and Novick, 1973; Sjöström et al., 1975; Pattee and Neveln, 1975; Pattee, 1976; Pattee et al., 1977; Kuhl et al., 1978; Soham et al., 1978, Novick et al., 1979). In addition, the ability of this organism to express competence is dependent upon the presence of a bacteriophage in either the prophage or vegetative state (Sjöström et al., 1973; Rudin et al., 1974).
This requirement for the presence of a phage for the expression of competence in *S. aureus* is unique among genetic transformation systems described for other bacterial species. The mechanism by which the phage confers competence to the host cell has been addressed previously (Sjostrom and Philipson, 1974); however, the role of the phage remains controversial. Therefore, this study reexamined the role played by bacteriophages in the acquisition of competence in *S. aureus*. 
LITERATURE REVIEW

Genetic transformation is a complex and biologically intriguing process that has been demonstrated in a number of bacterial species. The specific conditions that enable a population of cells to undergo transformation vary extensively with each organism in which a transformation system has been described. Therefore, a vast amount of literature has accumulated on genetic transformation (and transfection) in the last four decades. (For reviews see: Ravin, 1961; Schaeffer, 1964; Spizizen et al., 1966; Tomasz, 1969; Young and Wilson, 1972; Trautner and Spatz, 1973; Notani and Setlow, 1974; Zhdanor, 1977; and Benzinger, 1978).

This study was primarily concerned with the parameters that affect competence in Staphylococcus aureus; therefore, this literature review will deal mainly with the irreversible binding of DNA to bacterial surfaces and the transport of these molecules into the cell. The fate of the transforming (or transfecting) DNA within the recipient cell will not be considered except when pertinent to the penetration process.

Even within these limitations, the following literature review should not be considered to be exhaustive. Much of the published work on transformation is replete with inconsistencies and misinterpretations of other studies. In addition, discrepancies among authors in the expression of transformation (or transfection) frequencies make it nearly impossible to compare various procedures. Therefore, a certain amount of editorial license has been exercised in compiling this review.
History and Incidence of Transformation and Transfection

Historically, transformation was the first mode of genetic exchange to be described in bacteria. Griffith (1928) first observed the transformation of a nonencapsulated (i.e. nonvirulent) strain of Streptococcus pneumoniae (Diplococcus pneumoniae) to encapsulated (i.e. virulent) when injected into mice concomitantly with a heat-killed encapsulated strain. The ability to transform S. pneumoniae in vitro (Dawson and Sia, 1931) enabled this system to be instrumental in identifying DNA as the "transforming principle" and thus the genetic material (Avery et al., 1944). A very readable and humanistic account of this classical work can be found in a recent biography of O. T. Avery (Dubos, 1976).

Like the S. pneumoniae system, transformation in Hemophilus influenzae was first demonstrated using polysaccharide capsular types as the genetic character (Alexander and Leidy, 1951). Although antibiotic-resistance determinants were subsequently used in both of these systems (Hotchkiss, 1951; Alexander and Leidy, 1953), the fastidious growth requirements of these two organisms precluded the development of a sophisticated biochemical genetics system comparable to the one that was rapidly emerging in Escherichia coli by using conjugation. However, the demonstration of transformation in Bacillus subtilis (Spizizen, 1958) enabled the establishment of an easily manipulated transformation system based upon biochemical genetic determinants. Nevertheless, despite the difficulties involved with cultivation of the organisms and selection
of recombinants, *S. pneumoniae* and *H. influenzae*, along with *B. subtilis* remain the most extensively investigated transformation systems.

Bacterial species that have been shown to undergo transformation are listed alphabetically in Table 1. These transformation systems can be roughly divided into two classes. The first class consists of organisms in which competent cells arise "naturally" in populations of cells subjected to the proper cultural and environmental conditions. The second class consists of organisms that require, in addition to the proper cultural and environmental conditions, high concentrations of divalent cations, Ca$^{2+}$ being the most effective in most systems. This division is somewhat artificial in that divalent cations are required for some step in the transformation process of virtually all systems examined. However, the Ca$^{2+}$-induced systems require extremely high concentrations of Ca$^{2+}$, ranging from 25 mM in *E. coli* (Mandel and Higa, 1970; Cosloy and Oishi, 1973) to 100 mM in *S. aureus* (Rudin et al., 1974). In addition, the Gram-negative organisms that undergo Ca$^{2+}$-induced transformation generally also require a temperature shock from 0°C to 37°C or 42°C while in the presence of Ca$^{2+}$ for optimal results (Cohen et al., 1972; Cosloy and Oishi, 1973).

"Transformation" of bacteria with DNA isolated from high-frequency transducing lysates was first described by Kaiser and Hogness (1960). Their procedure involved exposure of galactose-negative *E. coli* K12 cells to isolated λdg DNA in the presence of vegetative λparticles; Gal$^+$ transformants were recovered. Subsequently, it was shown that vegetative λDNA isolated from a genetically marked phage could provoke
**Table 1. Incidence of transformation and transfection among bacterial species**

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Transformation Reference Method</th>
<th>Transfection Reference Method</th>
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<tbody>
<tr>
<td><em>Acholeplasma laidlawii</em></td>
<td>Juni and Janik (1969) Competent Cells</td>
<td>Liss and Maniloff (1972) Competent Cells</td>
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<tr>
<td><em>Acinetobacter calco-aceticus</em></td>
<td>Klein and Klein (1956) Competent Cells</td>
<td>Taketo (1972b) Ca$^{2+}$-induced</td>
</tr>
<tr>
<td><em>Aerobacter aerogenes</em></td>
<td>Holsters et al. (1978) Freeze-Thawing</td>
<td>Milani and Heberlein (1972) Competent Cells</td>
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<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>Page and Sadoff (1976) Competent Cells</td>
<td>Holsters et al. (1978) Freeze-Thawing</td>
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<tr>
<td><em>Azotobacter vinlandii</em></td>
<td>Gwinn and Thorne (1964) Competent Cells</td>
<td>Streips and Welker (1969) Competent Cells</td>
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<td><em>Bacillus licheniformis</em></td>
<td>Marmur et al. (1963) Competent Cells</td>
<td>Romig (1962) Competent Cells</td>
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<tr>
<td><em>Bacillus natto</em></td>
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<td><em>Bacillus steareothermophilus</em></td>
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<td><em>Bacillus subtilis</em></td>
<td>Spizizen (1958) Competent Cells</td>
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<td>Bacterial species</td>
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<td><em>Bacteriodes</em></td>
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<td>thetaioaomicron</td>
<td>Otero (1972)</td>
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<tr>
<td><em>Branhamella</em></td>
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<td>catarrhalis</td>
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<td><em>Escherichia coli</em></td>
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<td>Ca$^{2+}$-induced</td>
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<tr>
<td><em>Haemophilus</em></td>
<td>Alexander and Leidy (1951)</td>
<td>Competent Cells</td>
</tr>
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<td>influenzae</td>
<td></td>
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<td><em>Methylobacterium</em></td>
<td>O'Conner et al. (1977)</td>
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</tr>
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<td><em>Micrococcus</em></td>
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<td>lysodeikticus</td>
<td>Okubo and Nakamura (1968)</td>
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<td><em>Moraxella</em></td>
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<td>nonliquefaciens</td>
<td></td>
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<td><em>Mycobacterium</em></td>
<td>Bovre and Fröholm (1970)</td>
<td></td>
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<td>smegmatis</td>
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<td>tuberculosis</td>
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<td>Bacterial Species</td>
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<td>Proteus</td>
<td>van Rensburg (1971)</td>
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<td>mirabilis</td>
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<td>Khan and Sen (1967)</td>
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<td>Pseudomonas</td>
<td>Chakrabarty et al. (1975)</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-induced</td>
</tr>
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<td></td>
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<tr>
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</tr>
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</tr>
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<td>Bacterial Species</td>
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<td>Spirillum lipoferum</td>
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<td>Staphylococcus aureus</td>
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<td>Streptococcus pneumoniae</td>
<td>Griffith (1929)</td>
<td>Competent Cells</td>
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<td>Streptococcus sanguis (Group H streptococci)</td>
<td>Pakula et al. (1958)</td>
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<td>Thermoactinomyces vulgaris</td>
<td>Hopwood and Wright (1972)</td>
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<tr>
<td>Xanthomonas phaseoli</td>
<td>Corey and Starr (1957)</td>
<td>Competent Cells</td>
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a lytic infection when exposed to cells in the presence of a $\lambda$-helper phage carrying a different genetic marker (Kaiser, 1962). Similar results were subsequently obtained with phage P2 DNA and vegetative P2 particles (Mandel, 1967). The helper-phages in these two systems appear to play a dual role of altering permeability (Mandel, 1967) and providing cohesive ends (Kaiser and Imman, 1965; Strack and Kaiser, 1965) which evidently allow tandem association of the helper-phage DNA and the isolated phage DNA. Although the function of the helper-phage in these two systems appears to be identical, vegetative P2 particles cannot serve as a helper-phage for $\lambda$ DNA, nor can vegetative $\lambda$ particles serve as a helper phage for P2 DNA (Mandel and Berg, 1968). This incompatibility is probably due to nonhomologous cohesive ends. Phages P2 and 186 show a high degree of homology in their cohesive ends (Padmanabhan et al., 1974), and these two phages are compatible in heterologous phage infections (Mandel and Berg, 1968).

Romig (1962) successfully infected Bacillus subtilis cells with DNA isolated from bacteriophage SP3. This infection did not require the use of a homologous helper-phage but did require that the cells be naturally competent for transformation. Foldes and Trautner (1964) coined the term "transfection" to describe this process. "Transfection" has been used to describe infection of a cell with isolated viral nucleic acid regardless of the method by which the nucleic acid enters the cell.
Mandel and Higa (1970) successfully transfected *E. coli* with isolated λ and P2 DNA by exposing the cells to the DNA while treating the cells with cold CaCl₂. This transfection did not require the use of a homologous helper-phage; neither did it require that the cells be naturally competent for transformation. On the contrary, this Ca²⁺-shock procedure was shown to make *E. coli* "competent" for transformation (Cohen et al., 1972; Cosloy and Oishi, 1973) as well as for transfection. While the mechanism(s) of Ca²⁺-induced competence has not been elucidated, the development of this procedure for *E. coli* has supplied impetus to other investigators who have adapted this procedure to several other bacterial species that do not become naturally competent.

The bacterial species that have been shown to undergo transfection are also listed in Table 1. In addition to the naturally competent, Ca²⁺-induced competence, and helper-phage systems, several bacterial species can undergo transfection by a variety of other means.

While the bacterial cell wall is generally not considered to be a primary permeability barrier, the discussion to follow will establish that the cell wall does play a role in the adsorption and irreversible binding of exogenous DNA molecules to bacterial cells in species that demonstrate natural competence. However, it appears that many Gram-negative organisms will undergo transfection (or transformation) only if the outer membrane is partially or completely removed, or if the permeability is altered in some other way (calcium-shock, helper-phage).

Prior to the establishment of reliable transfection systems, infection of *E. coli* spheroplasts with urea-disrupted T₄ (Spizizen, 1957)
and DNA isolated from φX174 (Guthrie and Sinsheimer, 1960) and λ (Meyer et al., 1961) was accomplished. Although efficiencies of transfection among different methods in different studies are difficult to compare, spheroplasting appears to yield reproducibly high efficiencies of transfection, and these efficiencies can be further improved by the addition of protamine sulfate or spermidine (Henner et al., 1973). In addition, spheroplasts are receptive to several types of infectious RNA's (Knolle and Kaudewitz, 1962; Paranchych, 1963; Engelhardt and Zinder, 1964; Strauss, 1964; Benzinger et al., 1967) against which many other types of transformation and transfection generally discriminate. Therefore, this procedure has shown great utility in investigating the fate of foreign nucleic acids inside of bacterial cells (see review by Benzinger, 1978, for applications).

Because the recovery of transformants requires a colony-forming-unit, transformation of spheroplasts has not been well-documented. Chargaff et al. (1957) did report the transformation of E. coli spheroplasts; however, the frequency was very low, probably due to the low frequency of reversion to walled forms and the presence of DNases from lysed cells. Hirokawa and Ikeda (1966) and Tichy et al. (1968) reported efficient transformation of B. subtilis protoplasts. However, these two studies may have involved "quasi spheroplasts" which Tichy and Landman (1969) found to be highly transformable; these investigators found true lysozyme-induced protoplasts to be nontransformable. Miller et al. (1972) attributed this nontransformability of true B. subtilis protoplasts to an apparent lack of DNA affinity sites on the surface of...
the plasma membrane. Wilson and Bott (1970) also reported the inability to transfect *B. subtilis* protoplasts. However, Joenje et al. (1974) found that membrane vesicles isolated from competent *B. subtilis* will bind DNA. In addition, membrane vesicles isolated from competent cells bind more DNA than membrane vesicles isolated from noncompetent cells (Joenje et al., 1975).

Perhaps it is apropos to mention that with the advent of protoplast fusion technology (Schaeffer et al., 1976; Fodor and Alfoldi, 1976; Hopwood et al., 1977), reversion frequencies have increased to the level where transformation of protoplasts (and spheroplasts) has become feasible. Recently Chang and Cohen (1979) reported that treatment of *B. subtilis* protoplasts with polyethylene glycol can result in efficient uptake of plasmid DNA.

In addition to the complete spheroplasting of cells, transfection of *E. coli* has been accomplished by several other techniques that tend to disrupt the normal integrity of the cell wall and outer membrane. These include subjecting the cell to osmotic shock (Hofschneider, 1960; Rieber and Millan, 1971), freezing and thawing of the cells (Mackal et al., 1964; Dityatkin et al., 1972), growing the cells in glycine (Taketo, 1972a) or a hypertonic medium (Taketo and Kuno, 1969), and presenting the cells with DNA at acidic pH's (4.4 - 5.0) in the presence of protamine sulfate and sucrose (Osowiecki and Skalinska, 1974). The freeze-thaw technique of Dityatkin et al. (1971) was recently adapted for *Agrobacterium tumefaciens* by Holsters et al. (1978) who succeeded in transforming *A. tumefaciens* as well as transfecting the bacterium by
this method. However, these methods have not been well-documented and
will not be given further consideration unless the information can be
applied to other methods of transfection or transformation.

There has been considerable debate over the question of whether a
population of cells that is competent for transformation is also
competent for transfection, and vice versa. Aside from the transfection
of *E. coli* by using the helper-phage method (Kaiser and Hogness, 1960),
the original transfection systems used populations of cells that were
competent for transformation and merely substituted bacteriophage DNA
(Romig, 1962; Harm and Rupert, 1963). Because recombination with the
recipient genome is not required and the DNA fragments are genetically
homogenous in nature, transfection frequencies are often higher than
transformation frequencies. Many investigators consider transfection
to be a more accurate measure of the level of competence of a population
than transformation (Takagi et al., 1966; Bott and Wilson, 1967; Riva and
Polsinelli, 1968; Sjöström and Philipson, 1974; Notani and Setlow, 1974).
However, there have been reports of extremely large differences (1000X)
between the frequencies of transfection and transformation in *B. subtilis*
(Yasbin and Young, 1972; Yasbin et al., 1973) and group H streptococci
(Pakula et al., 1973; Parsons et al., 1973). Subsequently it was shown
that these differences in the *B. subtilis* system were due to prophage
induction during the expression of competence resulting in the loss of
the colony-forming ability of potential transformants (Garro and Law,
1974; Yasbin et al., 1975). Setlow et al. (1973) reported a similar
correlation between the transformation process and prophage induction in 
H. influenzae.

The distinction between transformation and transfection becomes increasingly difficult to distinguish when the nature of the DNA is examined. E. coli (Kaiser and Hogness, 1960), Rhizobium meliloti (Kondorosi et al., 1974) and S. aureus (Sjöström and Philipson, 1974) can be transformed for genetic characteristics with bacteriophage DNA isolated from high-frequency transducing lysates. Likewise, H. influenzae (Harm and Rupert, 1963; Boling et al., 1972) and B. subtilis (Romig, 1968; Rutberg et al., 1969) can be transfected with bacterial DNA isolated from lysogenic cells. Furthermore, transfection of B. subtilis with DNA isolated from certain bacteriophage will result in a lytic infection only if there is a recombination between separate phage genomes (Porter and Guild, 1978; Loveday and Fox, 1978).

There does not appear to be much conclusive evidence establishing that there is a difference; therefore, for the remainder of this review, competence for transformation and for transfection will be regarded as synonymous unless otherwise specified.

Genetic Control of Competence and Transformation

The expression of competence and the subsequent integration of new genetic material obviously has a genetic basis. Generally speaking, competence is only demonstrated in certain strains of a species, and strains that exhibit natural competence occasionally give rise to variants that are not transformable. This phenomenon was first
recognized during the classical studies of Avery et al. (1944). The loss of the ability to undergo transformation "breeds true" in that the progeny of a nontransformable variant are not transformable (Young and Spizizen, 1961). However, there has been no precise genetic analysis of the transformation process in any of the systems examined.

Because transformation is a multistep process, a genetic lesion may affect a variety of cellular functions. However, mutants affecting transformation can be divided into two very large categories: (1) those involving DNA binding and uptake and (2) those involving some aspect of the recombinational process.

Mutants classed in the first category can be referred to as geneti­cally noncompetent (Com\(^-\)). This phenotype can be manifested by a variety of defective processes; these include the loss of the ability to produce or bind a competence factor (Tomasz, 1965a; Streips and Welker, 1971), the loss of the ability to irreversibly bind DNA (Caster et al., 1970), or the loss of the ability to produce or respond to an autolytic enzyme (Young and Spizizen, 1963).

Mutants classed in the second category can be referred to as recombinationally defective (Rec\(^-\)). Again, this phenotype can be manifested by a variety of defective processes. Certain rec mutants of H. influenzae appear to be deficient in forming normal host-donor complexes but appear to be deficient in integration (Notani et al., 1972; Postel and Goodgal, 1972). Similar classes of mutants have been described for B. subtilis (Dubnau et al., 1973).
Finally, information derived from one organism is difficult to apply to the transformation system of another organism. For example, a deficiency in an adenosine triphosphate-dependent deoxyribonuclease (ATP-dependent DNase) in \textit{S. pneumoniae} inhibits the process of transformation (Vovis, 1973). In \textit{E. coli}, a deficiency in an ATP-dependent DNase is a necessity for successful transformation of chromosomal markers (Cosloy and Oishi, 1973). However, a deficiency in a similar enzyme in \textit{H. influenzae} appears to have no effect on the ability to undergo transformation (Wilcox and Smith, 1975).

\section*{Natural Competence}

The phenotypic expression of competence requires a number of coordinated conditions that vary extensively with the system being investigated. The vast majority of available information concerning competence has been derived from studies involving \textit{S. pneumoniae}, \textit{H. influenzae}, \textit{B. subtilis} and the group \textit{H} streptococci. These bacteria all demonstrate natural competence. Thus, most of the following discussion will focus on the state of competence in these organisms.

\section*{Expression of competence}

Many investigators consider the competent state to be a pathological condition arising from "unhealthy" cells accumulating in the culture. This view is somewhat supported by the fact that competence can be demonstrated in \textit{B. subtilis} (Young and Spizizen, 1961) and \textit{H. influenzae} (Alexander et al., 1954) only toward the end of the log phase and into the stationary phase of growth; this period is normally associated with
metabolic latency. Furthermore, both *B. subtilis* (Anagnostopoulos and Spizizen, 1961) and *H. influenzae* (Spencer and Herriott, 1965) can be induced to show higher levels of competence if the cells are removed from a medium that supports growth and inoculated into a medium that does not support growth.

Dooley et al. (1971) described a series of physiological changes that occur in *B. subtilis* during the development of competence. These changes include a decrease in DNA synthesis and a lowered ratio of RNA synthesis to protein synthesis. These changes in macromolecular content result in a decrease in buoyant density of competent cells and allow the isolation of a population of cells enriched for competent cells by centrifugation in a Renografin gradient (Hadden and Nester, 1968). However, the development of competence in *B. subtilis* is inhibited by chloramphenicol (Akrigg et al., 1967). Finally, competent cells are resistant to penicillin (Nester and Stocker, 1963) and remain resistant only as long as they remain competent (Nester, 1964). It has been suggested that competence in *B. subtilis* is associated with sporulation. Young and Spizizen (1961) isolated several mutants that could not undergo transformation and found them also to be defective in sporulation. However, the correlation of competence to sporulation must be made with caution because many asporogenous mutants show no decrease in the ability to undergo transformation (Schaeffer, 1964). In addition, *B. subtilis* growing in a chemostat under conditions that are not conducive to sporulation exhibit a significant level of competence (Lopez et al., 1975). However, the actual point in the sporulation process that is
blocked genetically or by environmental manipulation is probably relevant to this argument.

The transfer of \textit{H. influenzae} from a growth-supporting to a growth-arresting medium results in the induction of high levels of competence (Spencer and Herriott, 1965); this suggests that nutritional depletion results in metabolic arrest that is manifested in the expression of competence. However, the addition of cyclic adenosine 3',5'-monophosphate (cyclic AMP) can induce competence in \textit{H. influenzae} in a growth-supporting medium (Wise et al., 1973). Cyclic AMP has been shown to relieve catabolite repression (Pastan and Perlman, 1970). Therefore, it is probable that the nutritional shift procedure has a similar effect. However, Zoon et al. (1975) contend that catabolite repression alone is not a sufficient condition to account for competence in \textit{H. influenzae} induced by nutritional shift.

Competence in the group \textit{H} streptococci is associated with log growth (Pakula and Walczak, 1963), a period of active metabolism and uniformity of cell physiology. However, Horne and Perry (1975) detected a reduction in the rates of RNA and peptidoglycan synthesis during the induction of competence in strain Wicky by competence factor obtained from strain Challis.

Competence in \textit{S. pneumoniae} is usually associated with log growth but appears to be more dependent upon cell density than upon the "closeness" to stationary phase (Tomasz, 1965b). Although competent \textit{S. pneumoniae} have been reported to synthesize less DNA than noncompetent cells (Ephrussi-Taylor and Freed, 1964), another report contends that
there is no difference in macromolecular biosynthesis in competent and noncompetent cells (Tomasz, 1968). Also, competent *S. pneumoniae* remain sensitive to penicillin (Tomasz, 1964a).

Perhaps because of the ease of manipulation, the effect of nutrients and medium components has been examined extensively in connection with the expression of competence. The formulation of chemically defined media that allow the development of competence in *B. subtilis* (Wilson and Bott, 1968), *H. influenzae* (Herriott et al., 1970), *S. pneumoniae* (Tomasz, 1964b), and the group H streptococci (Lawson and Gooder, 1970) has facilitated the examination of various media components for their effects on the expression of competence. Although the presence of certain media components may result in a variety of physiological effects that may affect competence, this discussion will focus only on those that are particularly germane to the study to be presented.

The development of competence in *H. influenzae* by using the nutritional shift procedure requires that specific nutrients be present in the growth-phase medium. The specific nutrients found to produce maximum competence were identified as lactate and inosinic acid (Ranhand and Herriott, 1966). These compounds are required for the synthesis of an active site that is converted to the DNA-binding site in the nongrowth-phase (Ranhand, 1969). This site is irreversibly destroyed by treatment with periodate (Ranhand and Lichstein, 1966).

Wilson and Bott (1968) examined the effect of various amino acids for the ability to stimulate or inhibit the development of competence in *B. subtilis*. All of the amino acids found as major components of the
cell wall were inhibitory; however, the implications of this finding were not pursued. Young (1965) observed that *B. subtilis* cells grown in a medium that allows the development of competence have twice as much galactosamine associated with the cell wall as cells grown in a medium that does not support the development of competence. In addition, he found that nontransformable mutants have less galactosamine associated with their cell walls than wild-type organisms. Polsinelli and Barlati (1967) demonstrated that competence can be inhibited by treatment with periodate.

Tomasz et al. (1971) demonstrated that the presence of choline, a component of the teichoic acid, in the growth medium was essential for the development of competence. Substitution of the choline with ethanolamine resulted in the incorporation of the ethanolamine into the teichoic acid, but these cells could not undergo transformation. This inhibition could be reversed by transferring the ethanolamine-containing cells to a medium containing choline.

Although not essential, the presence of serum in the growth medium of both *S. pneumoniae* and the group H streptococci greatly stimulates the frequency of transformation. While serum albumin and phosphate ions could substitute for whole serum in the *S. pneumoniae* system (Hotchkiss and Ephrussi-Taylor, 1951), these compounds could not completely replace the whole serum in the group H streptococci system (Pakula, 1965a).

Inhibitors of macromolecular biosynthesis have been used as tools to establish the biosynthetic status of cells during the development of competence. Although the studies involving DNA and RNA inhibition are
generally inconclusive, investigators agree that protein synthesis is essential for the expression of competence in all the systems examined in detail (Akrigg et al., 1967; Tomasz, 1970; Spencer and Herriott, 1965). In some systems, the requirement for protein synthesis can be traced directly to a specific protein that is essential to the acquisition of the competent state; in other systems, the specific protein(s) involved has not been identified.

During the development of competence in *S. pneumoniae* (Tomasz and Hotchkiss, 1964) and the group Η streptococci (Pakula and Walczak, 1963) an extracellular protein is produced that is essential for the expression of competence. Although these extracellular proteins are referred to by a variety of terms in the literature (competase, activator, competence-provoking factor), in this review they will be referred to as competence factors.

The competence factor produced by *S. pneumoniae* is a protein with a molecular weight of approximately 10,000 daltons; it is sensitive to inactivation with proteolytic enzymes and appears to be distinct from the pneumococcal autolytic activity (Tomasz and Mosser, 1966). This competence factor appears to induce its own production (Tomasz, 1966); thus competence is spread "epidemically" through a culture. It has limited interspecific activity in that some streptococci closely related to *S. pneumoniae* can be made minimally competent by reaction with the pneumococcal competence factor (Tomasz and Mosser, 1966). The mechanism by which the competence factor regulates the expression of competence in
S. pneumoniae is beginning to be revealed. The following is a brief synopsis of the available information.

Vaccines prepared from competent, but not noncompetent, *S. pneumoniae* cells can invoke the formation of antibodies in rabbits that will inhibit the binding of DNA to competent cells (Nava et al., 1963). In addition, these antibodies prepared against competent cells can inhibit the acquisition of competence by noncompetent cells in the presence of the competence factor (Tomasz and Beiser, 1965). This suggests that the cell wall-bound competence factor might be the competence-specific antigen. However, the exact acceptor site for the competence factor has not been determined. Rejholcova et al. (1974) found that teichoic acids isolated from *S. pneumoniae* could inhibit the induction of competence by the competence factor. These investigators also demonstrated that several phytohemagglutinins as well as glucosamine and galactosamine could inhibit the development of competence in the presence of competence factor (Kohoutova and Kocourek, 1973). These findings seemed to suggest that the aminosugar-containing teichoic acid forms a part of the competence factor receptor site. However, more recently, Horne et al. (1977) showed that a repertoire of nonspecific substances can inhibit the development of competence in the presence of the competence factor. Moreover, Ziegler and Tomasz (1970) showed that when cells were exposed to competence factor and subsequently converted to spheroplasts with pneumococcal autolytic enzyme, 71% of the competence factor could be recovered associated with the spheroplast membrane. Horne et al. (1977) succeeded in isolating a protein from the membrane of *S. pneumoniae*.
that inhibited the competence-inducing activity of the competence factor; this membrane protein did not appear to have proteolytic activity, and presumably inactivates the competence factor by specifically binding with it. Another unique property of the pneumococcal competence factor is that cells treated with the competence factor under the appropriate nutritional and environmental conditions can be converted to spheroplasts (as determined by microscopic observation) (Seto and Tomasz, 1975a). This procedure results in the loss of the ability of the protoplast to bind DNA; however, cell membrane complexes prepared from competent cells demonstrate an ability to bind DNA. Cell membrane complexes prepared from noncompetent cells do not demonstrate the ability to bind DNA (Seto et al., 1975). Finally, a proteinaceous compound can be isolated from cell membrane complexes from competent cells, but not from noncompetent cells, that has the ability to bind DNA (Seto and Tomasz, 1975b).

The information presented above seems to be consistent with the following model concerning the development of competence in S. pneumoniae. S. pneumoniae cultivated under the proper conditions synthesizes and liberates a certain amount of competence factor during log growth. This competence factor binds to cells in the culture. The binding of the competence factor to a receptor site that appears to be associated with the cell membrane might result in the liberation of preformed competence factor or the induction of the synthesis of competence factor by the cell. The binding might also result in the liberation or the induction of the synthesis of an autolytic enzyme that unmasks DNA
binding sites on the cell membrane. The substitution of ethanolamine for choline in the growth medium results in the incorporation of ethanolamine into the teichoic acid; the ethanolamine renders the cell wall insensitive to the autolytic enzyme, and thus the cell cannot bind DNA.

In the group H streptococcus system, the role of the competence factor has not been as thoroughly investigated as in the pneumococcal system. The streptococcal competence factor is a small, highly basic polypeptide with a molecular weight of about 5,000 daltons and can be inactivated by treatment with proteolytic enzymes (Osowiecki et al., 1969). This polypeptide is found in the medium of competent cultures of the transformable strain Challis; incubation of the nontransformable strain Wicky in this growth medium will result in the acquisition of competence by strain Wicky (Pakula and Walczak, 1963). Pakula (1965b) reported that treatment of competent Challis and Wicky cells (after being made competent with competence factor from strain Challis) with antibodies prepared against competent Challis and Wicky cells inhibited the process of transformation. He also reported (Pakula, 1967) that antibodies prepared against noncompetent Challis and Wicky cells could inhibit the induction of competence by competence factor. Therefore, there must be present a specific binding site for the competence factor on the cell surface. Deddish and Slade (1971) reported the presence of a proteinaceous receptor site on the cell wall of competent strain Challis. However, their cell wall preparations were not shown to be free of contaminating cell membrane material. Perry (1974) obtained
evidence, using spheroplasts of strain Challis, that the competence factor binding site is associated with the cell membrane. However, Perry's cell membranes were not shown to be free of cell wall material. Finally, Ranhand (1974) found that an autolytic activity is associated with the development of competence in strain Wicky after being treated with competence factor from strain Challis. Although the streptococcal system is not as well characterized as the pneumococcal system, the similarities are quite apparent.

There has been much confusion concerning the presence of a competence factor associated with the development of competence in B. subtilis. Akrigg et al. (1967) demonstrated that a factor was released from osmotically shocked competent cells that stimulated the development of competence in noncompetent cells. The subsequent isolation of this competence-inducing activity by ion-exchange chromatography resulted in a fraction that contained autolytic activity as well as the competence-inducing activity (Akrigg and Ayad, 1970). Young and Spizizen (1963) had previously reported an increase in autolytic activity of B. subtilis at the time of maximum competence; poorly transformable cells did not show this increase in autolytic activity when grown under the same conditions. The autolytic activity was heat labile and was presumed to be enzymatic in nature. Therefore, this autolytic activity might be considered to be a competence factor, but it probably remains closely associated with the cell during normal growth and the development of competence.
However, Joenje et al. (1972) reported that culture fluids from competent *B. subtilis* cells could stimulate the development of competence in noncompetent cells. This activity was not inactivated by trypsin or Pronase but could be removed from the culture fluid by passage through cellulose nitrate. Goldsmith et al. (1970) could not demonstrate the presence of any type of competence factor that could be transferred from competent to noncompetent cells growing in a mixed culture and distinguishable by genetic markers. In addition, treating the competent cells with Pronase (100 to 300 μg/ml) did not result in a decrease in transformability, although the presence of proteinaceous sites that are not accessible to Pronase could not be ruled out.

In addition to *B. subtilis*, competence in *B. stearothermophilus* has been reported (Streips and Welker, 1969). A competence factor that is released into the medium by competent *B. stearothermophilus* has been reported, but not intensively investigated. It does appear to be inactivated by papain but not by trypsin (Streips and Welker, 1971).

In the *H. influenzae* transformation system, a proteinaceous competence factor has not been detected. However, there has been one report that filtrates from competent cells can stimulate the development of competence in cells that have not yet become competent. The factor responsible for this stimulation is dialyzable but not sensitive to deoxyribonuclease (DNase), ribonuclease (RNase), or proteolytic enzymes. In addition, the stimulation was inhibited by treating the cells with puromycin (Barnhart, 1967). From these data, it is not unreasonable to propose that this "competence factor" might be cyclic AMP that has been
liberated from some of the cells, perhaps by autolysis, as the cells approach stationary phase.

Antibodies prepared against competent *H. influenzae* inhibit the binding of DNA to competent cells. Antibodies prepared against non-competent cells do not inhibit this binding (Bingham and Barnhart, 1973). The specificity of these antigenic sites has not been determined, but Zoon and Scocca (1975) reported several changes in the cell membrane of *H. influenzae* during the development of competence. These changes include an increase in the amount of lipopolysaccharide (LPS) and an increase in the carbohydrate content of the LPS. In addition, several distinct proteins were also found to be associated with a crude extract of the cell envelope during the development of competence.

**Binding and uptake of DNA**

The general features of the processes involved with binding and uptake of DNA molecules by naturally competent cells have been incorporated into several recent reviews on recombination in prokaryotic organisms (Dubnau, 1976; Fox, 1978; Low and Porter, 1978). Therefore, the information presented on this subject in this review will be limited.

Certain artifacts inherent in studies involving the binding and uptake of DNA should probably be identified. The two main concerns involve the identification of irreversible binding of DNA and the incorporation of radioisotopic labels into other polymers.

The generally accepted definition of a competent cell is one that can irreversibly bind and take up DNA molecules. Haseltine and Fox (1971) have described a nonspecific reversible interaction of DNA
molecules with noncompetent cells that appears to result in the inactivation of the transforming ability of these molecules. It is important, then, to differentiate between reversible and irreversible binding. The use of DNase-insensitivity as an index of irreversible binding has been used extensively; in the majority of these cases, DNase-insensitivity also carries the connotation of consequential uptake. However, the following cases suggest that perhaps DNase-insensitivity should not be used as the sole criterion for inevitable uptake.

Erickson et al. (1969) demonstrated that DNA that had been exposed to competent _B. subtilis_ cells for a sufficient period of time to render it DNase-insensitive could be inhibited from entering the cell by the addition of antibodies prepared against single-stranded DNA. The DNA molecules could be recovered from this system in the form of a DNA-antibody-membrane complex. Strauss (1970) found that DNA that had been rendered DNase-insensitive by the reaction with competent _B. subtilis_ cells can be inhibited from transforming the cells by the addition of cyanide to the system for up to 6 minutes after the time that the DNA becomes DNase-insensitive. Finally, Harris and Barr (1969) showed that the amount of labeled transforming DNA that could be recovered from cells after the DNA had become DNase-insensitive could be decreased substantially by simply washing the cells.

The other artifact with which investigators must be concerned involves the nonspecific incorporation of isotopes into other cell polymers. This is particularly a problem if $^{32}$P-labeled DNA is extracted from Gram-positive organisms. Young and Jackson (1966) showed that $^{32}$P can be
incorporated into the cell wall teichoic acid and that conventional methods of DNA preparation (phenol extraction, ethanol precipitation) do not completely remove it from the DNA.

Cells that demonstrate natural competence have a predilection for double-stranded DNA over other forms of nucleic acids. Single-stranded DNA has been shown to be effective in the transformation of *B. subtilis* and *H. influenzae* if an acidic pH is used and a chelating agent is present (Postel and Goodgal, 1967; Tevethia and Mandel, 1970; Tevethia and Mandel, 1971). *S. pneumoniae* appears to be able to utilize single-stranded DNA at an efficiency of about 0.5% that of double-stranded, using conditions of a normal transformation procedure (Miao and Guild, 1970).

The efficiency of binding and uptake of circular DNA has not been studied extensively in systems that demonstrate natural competence. Canosi et al. (1978) examined various conformational species of circular DNA that had been purified by electrophoresis for their effectiveness in transforming *B. subtilis*. This circular DNA was the pC194 plasmid from *S. aureus* that had been transferred to *B. subtilis* and then reisolated. These investigators found the monomeric form of the plasmid (either closed-circular or open-circular forms) to be very inefficient in transforming *B. subtilis*. However, multimeric forms of the plasmid were 100-1000 times more efficient than the monomeric forms. If the monomeric forms were converted into multimeric forms by treatment with *HindIII* and ligase, the resulting multimeric forms were highly efficient.
Thus, the size of the DNA molecule appears to play a role, although the actual processing of the molecule was not followed in this study.

Most procedures for preparing bulk DNA for transformation result in extensive shearing of the molecules. Therefore, a typical transformation reaction generally contains DNA molecules of varying lengths. However, there seems to be a minimum effective size. Morrison and Guild (1972) could not demonstrate transformation of \textit{B. subtilis} with DNA molecules smaller than $1.2 \times 10^6$ daltons. Because the assay for this study was the transformed cell and binding and uptake of smaller molecules were not measured directly, this minimum molecular weight may reflect the requirement of any of a number of steps in the transformation process.

It appears that most competent cells will bind and take up either heterologous or homologous DNA. For many years it was assumed that heterologous and homologous DNA were taken up with equal efficiency and that restriction enzymes or lack of homology with the recipient DNA prevented expression of the heterologous donor DNA. However, several recent reports suggest that there might be a certain amount of discrimination between types of DNA at the level of binding and uptake. Piechowska et al. (1975) reported that \textit{B. subtilis} does not appear to take up phage T6 DNA, while it does seem to take up nonglucosylated T6 DNA and also \textit{E. coli} DNA. Scocca et al. (1974) reported that DNA from \textit{E. coli}, \textit{Xenopus laevis}, calf thymus, or salmon sperm could not compete with homologous DNA in the \textit{H. influenzae} transformation system; however, DNA from the closely related \textit{H. parainfluenzae} could
compete with the homologous DNA. These results seem to suggest that there might be a mechanism that inhibits (or at least discourages) the entrance of foreign DNA at the cell surface. This concept could help to change the current attitude that seems to regard transformation as a laboratory phenomenon and not an effective mode of genetic exchange between bacteria in nature. In fact, a recent report suggests that *B. subtilis* can be effectively transformed in a soil environment (Graham and Istock, 1978).

Although transformation with RNA has not been well-documented, there have been a few reports of a transient transformation using isolated RNA (Kirtikar and Duerksen, 1968) or a DNA:RNA hybrid (Hurwitz et al., 1964; Evans, 1964). Confirmations of these early reports have not been forthcoming.

The number of DNA binding sites per cell ranges from about 5 in *H. influenzae* (Scocca et al., 1974) to 20 to 50 in *B. subtilis* (Singh, 1972) and 30 to 80 in *S. pneumoniae* (Fox and Hotchkiss, 1957). The binding of double-stranded DNA to the surface of the competent cell appears to be noncovalent in nature because treatment of *S. pneumoniae* with guanidine chloride quantitatively releases the bound DNA (Morrison and Guild, 1973).

The actual uptake of the bound DNA appears to be quite similar in *B. subtilis* and *S. pneumoniae*. In both of these systems, the high molecular weight double-stranded DNA is cleaved into smaller double-stranded fragments. These fragments are approximately 10–20 kilobases long in *B. subtilis* (Dubnau and Cirigliano, 1972) and 8–9 kilobases long in *S. pneumoniae* (Morrison and Guild, 1973). An enzyme that is
capable of cleaving high molecular weight double-stranded DNA into fragments of approximately $1 \times 10^6$ daltons or more has been isolated from *B. subtilis* cells. This enzyme is released from the cell during protoplast formation; therefore, it is probably associated with the periplasmic space (Scher and Dubnau, 1976).

After the production of double-stranded fragments, the fragments are taken up by the competent cell. During the uptake process, one strand is degraded resulting in the appearance of a single-stranded DNA inside the cell (Lacks, 1962; Piechowska and Fox, 1971). At this point, the donor markers cannot be recovered from the cell in the form of genetically active transforming DNA; therefore, this period is referred to as the eclipse (Venema et al., 1965; Ghei and Lacks, 1967). This apparent inactivation of the donor DNA is probably a reflection of the ineffectiveness of small single-stranded DNA in conventional transformation procedures.

In *H. influenzae*, the transforming DNA does not become completely converted to a single-stranded form upon uptake (Notani and Goodgal, 1966); however, single-stranded regions are detected which seem to be necessary for successful integration (Sedgwick and Setlow, 1976).

The binding and uptake of DNA has not been studied extensively in the group H streptococci transformation system. However, Raina and Ravin (1977) could not detect any single-stranded donor DNA in cells lysed after the donor DNA had been taken up.
Expression of competence

The refractility of *E. coli* and many other important Gram-negative bacteria to transforming and transfecting DNA without the physical removal of the cell envelope has been circumvented by treating the cells with high concentrations of calcium ions. The use of this method for transfecting members of the *Enterobacteriaceae* has been recently reviewed (Benzinger, 1978). The method was initially described for transfecting *E. coli* with λ and P2 DNA and involves treating the cells at 0°C for 20 min with CaCl$_2$ (10–50 mM) in the presence of DNA, followed by incubation at 37°C for 20 min (Mandel and Higa, 1970). Slight variations of this procedure are used by different investigators for the transfection of *E. coli* with phage nucleic acid.

This procedure was readily modified to achieve the transformation of *E. coli*, using purified plasmid DNA (Cohen et al., 1972) and chromosomal DNA (Cosloy and Oishi, 1973). The transformation of *E. coli* with chromosomal DNA requires the use of a recipient that is defective in the ATP-dependent DNase (recBC). This requirement evidently reflects the enzymatic nature of the recBC gene product and the activation of other recombinational systems in the absence of the recBC gene product (Oishi and Irbe, 1977).

While the competence in *E. coli* is induced by treatment of the cells with Ca$^{2+}$, cells cultivated under certain growth conditions seem to be more responsive to the treatment. Taketo (1974) found that cells
harvested from nutrient broth during the late log phase demonstrated higher levels of transfection than cells harvested during other growth phases. He also found that cells cultivated at temperatures below 25°C responded very poorly to transfection. However, using transforming DNA, Irbe and Evans (1976) found that cells harvested in early log phase were more responsive. This discrepancy might reflect other variables in cultural conditions because Irbe and Evans (1976) also found that cells grown on glucose were more responsive than cells grown on succinate, and cells grown in brain heart infusion transformed poorly.

**Binding and uptake of DNA**

Taketo (1972b) found that *E. coli* was more efficiently transfected with double-stranded phage DNA than with single-stranded phage DNA or phage RNA; however, it is not possible to tell from his work if the inefficiency of single-stranded DNA or RNA is due to decreased binding and uptake or to ineffective expression of the genetic material. Sabelnikov et al. (1975) estimated that a Ca\(^{2+}\)-treated *E. coli* cell could bind approximately \(6 \times 10^8\) daltons of chromosomal DNA per cell. They found that the irreversible binding of DNA appeared to be dependent upon the presence of Ca\(^{2+}\), and most of the DNase-insensitive DNA was found associated with the cell membrane. However, Taketo (1978) isolated a number of mutants with defective surface lipopolysaccharide structure that showed a decrease in the level of transfection, and several mutants with defective envelope lipoprotein showed an increased response to transfection. This suggests that the outer membrane may also play a role in the binding of DNA.
The actual mechanism by which Ca\(^{2+}\) induces the binding and uptake of DNA by \textit{E. coli} is not known. Most of the bacteria that exhibit natural competence require the presence of divalent cations at some step during the transformation process. However, the concentrations of cations required by these transformation systems are relatively low (less than 1.0 mM). These concentrations are suggestive of those needed for activation of a specific protein, perhaps a nuclease, on the cell surface. However, the information regarding the presence of nucleases and their activation during transformation is fragmentary. The high concentrations of Ca\(^{2+}\) required by \textit{E. coli} for successful transfection or transformation is suggestive of gross physical alterations of the molecular components involved. Because there is very little experimental evidence in support of any hypothesis, all models regarding the uptake of Ca\(^{2+}\)-treated \textit{E. coli} cells must be regarded as merely conjecture. Therefore, the following brief discussion focuses on hypothetical situations.

The high concentrations of Ca\(^{2+}\) might neutralize the charge on the cell surface and perhaps even bind the negatively charged DNA molecule to the surface of the cell. This binding might even be in the form of a precipitate; Graham and van der Eb (1973) have shown that precipitation of adenovirus 5 DNA with calcium chloride results in increased infectivity of the isolated DNA for human KB cells. This "precipitation" may also impart to the DNA molecule a configuration necessary for the uptake of the DNA.
The information derived from the use of Ca\(^{2+}\) to induce fusion of membrane vesicles might help to elucidate the role of Ca\(^{2+}\) in the induction of competence in *E. coli*. Treating artificially prepared membrane vesicles with Ca\(^{2+}\) (1-2 mM) results in increased permeability of the vesicle as measured by Na\(^+\) efflux, aggregation of vesicles, and fusion of the aggregated vesicles (Papahadjopoulos et al., 1977). Some or all of these apparently coordinated phenomena might be due to phase transitions of the membrane phospholipids from a fluid to a crystalline state; these phase transitions can be induced by treating membrane vesicles with Ca\(^{2+}\) (Jacobson and Papahadjopoulos, 1975). It is conceivable that reaction of Ca\(^{2+}\) with *E. coli* membrane phospholipids might result in a membrane configuration conducive to the uptake of the large negatively charged DNA molecules. The reaction of Ca\(^{2+}\) with membrane phospholipids liberates a heat of crystallization (Papahadjopoulos et al., 1977), which might explain the requirement for a low temperature (0°C) during the initial steps of DNA binding and uptake. However, a decrease in temperature can also generate a phase change in membranes (Steim et al., 1969). Therefore, the low temperature may also augment the effect that Ca\(^{2+}\) has on the configuration of the membrane.

Finally, it is possible that the high concentration of Ca\(^{2+}\) simply inactivates a protein in the cell membrane that normally inhibits the uptake of DNA.
Competence in *Staphylococcus aureus*

Genetic manipulations in *S. aureus* were made possible by the demonstration of generalized transduction (Ritz and Baldwin, 1958; Morse, 1959) and specialized transduction (Novick, 1967) in this bacterium. Although this technique proved to be of great utility in fine-structure mapping (see review by Egan, 1972), the limited size (Lacey, 1975) and apparent homogeneity (Pattee et al., 1968b) of the staphylococcal transducing fragments limited its usefulness. Therefore, a vast amount of time and energy was expended trying to develop a method by which *S. aureus* could be transformed.

The first report of transformation of *S. aureus* appeared in 1959 (Imshenetskii et al., 1959). These investigators reported the apparent transformation of a streptomycin-sensitive strain of *S. aureus* with DNA isolated from a mutant that had been selected for increased resistance to the antibiotic. However, this report was lacking in experimental detail and contained incomplete citations.

Riggs and Rosenblum (1969) successfully transfected *S. aureus* with phenol extracted phage DNA. However, these researchers found it necessary to treat the cells with lysostaphin in order to make the cells permeable to the DNA. The efficiency of transfection was very low (2 per $10^7$ cells) and the recovery of viable cells from this procedure was difficult. Therefore, this method was not pursued in an attempt to find a transformation protocol for *S. aureus*.

Nestoresco et al. (1968) reported that DNA isolated from enterotoxigenic strains of *S. aureus* could be used to transform cells of
S. aureus strain 73 which are not enterotoxigenic. However, successful transformation was dependent upon the addition of a temperate phage that had been propagated on the donor strain. From these experiments, 2 out of 120 isolates were obtained that produced enterotoxin. The possibility that transduction could have been responsible for these genetic exchanges was discounted on the basis that there were no enterotoxigenic isolates in a control group that was infected with phage, but no DNA. However, this control group consisted of only 8 isolates. These investigators also used autoradiography with \(^3\)H-thymidine to detect DNA uptake. However, their procedure involved several intermediate platings; therefore, the resultant incorporation was probably due to digestion of the labeled DNA by extracellular DNases and incorporation of the labeled nutrients into new cellular material.

Nomura et al. (1971) reported the transformation of nonhemolytic strains of S. aureus to hemolytic by DNA extracted from hemolytic strains. These transformations were performed in a hypertonic medium, using DNA extracted by the Marmur procedure (Marmur, 1961). Although Lindberg et al. (1972) were unable to transform S. aureus 8325 with DNA prepared by the Marmur procedure, the fact that the transformations were performed in a hypertonic medium might have facilitated the uptake of DNA.

Using an adaptation of the procedure developed by Mandel and Higa (1970) for the transfection of E. coli with \(\lambda\) DNA, Sjöström et al. (1972) succeeded in transfecting S. aureus 8325 with phenol extracted DNA isolated from phage 80a. This procedure required the treatment of
the staphylococcal cells with 0.1 M CaCl₂ prior to the addition of DNA. This procedure was subsequently used by the same investigators to transform genetically marked derivatives of 8325 with phenol extracted wild-type DNA (Lindberg et al., 1972). In the same study, the transformation of plasmid-borne determinants was also observed, using bulk DNA isolated from an 8325 derivative carrying the PI₂₅₈ plasmid.

The reproducibility of this procedure was confirmed by Pattee and Neveln (1975) who used the technique for locating genetic determinants in various linkage groups on the staphylococcal chromosome.

A more complete study of the physiological and genetic requirements for the expression of Ca²⁺-induced competence for transfection (Sjöström et al., 1973) and transformation (Rudin et al., 1974) in S. aureus established that competence for transfection and for transformation appeared to be identical. Cells responded to the Ca²⁺ treatment only if they were harvested in early log phase. Optimum results were obtained if the cells were exposed to the DNA at a pH around 7.0 and a temperature around 30°C. Unlike Ca²⁺-induced competence in E. coli, the transfection and transformation in S. aureus did not require a temperature shock. The optimum concentration of Ca²⁺ was found to be 0.1 M; Mg²⁺ or Ba²⁺ could be substituted for Ca²⁺, but these cations were not as effective as Ca²⁺. In addition, protein synthesis was required for the expression of competence.

The wild-type strain 8325 is lysogenic for the prophages φ11, φ12, and φ13 (Novick, 1967). A derivative that has been cured of these prophages (8325-4) is not able to demonstrate competence for
transfection (Sjöström et al., 1973). Lysogenization of 8325-4 with each of the prophages isolated from the wild-type bacterium resulted in the derivatives 8325-4(φ11), 8325-4(φ12), and 8325-4(φ13). When these single lysogens were tested for the ability to undergo transfection, only 8325-4(φ11) demonstrated this ability. Thus, it was concluded that the prophage is necessary for the expression of competence in strain 8325 (Sjöström et al., 1973). Besides φ11, only lysogens carrying the closely related phages 83A (Sjöström and Philipson, 1974) and φ14 (Rudin and Lindberg, 1975) could demonstrate competence. The origin of the latter phage is unclear, but phages φ11 (Novick, 1967), 83A (Wentworth, 1963) and φ14 (Rudin and Lindberg, 1975) all belong to the serological group B; therefore, they all are morphologically related.

Sjöström et al. (1973) also demonstrated that vegetative φ11 could confer competence to 8325-4. This infection resulted in lysogenization of the competent cell if it was recovered as a transformant (Rudin et al., 1974). In addition, superinfection of a φ11-lysogen with vegetative φ11 particles resulted in an enhancement of the transfection frequencies observed (Sjöström et al., 1973). Therefore, in addition to conferring competence in the prophage state, φ11 could confer competence upon infection of nonlysogenic cells with the consequential lysogenization (helper-phage), and φ11 could enhance the level of competence in a culture of lysogenic cells upon superinfection. Fig. 1 is a diagrammatic representation of the modes by which these investigators concluded that φ11 could confer competence.
Fig. 1. Diagrammatic representation of 3 cells exhibiting the different modes by which φ11 confers competence as described by Sjöström and Philipson (1974). The larger circle represents the staphylococcal cell, and the smaller circle represents the bacterial chromosome. The phage genome is represented by the genetic material between the hash marks on the bacterial chromosome in the cells on the right and left and by the genetic material in the process of integrating into the bacterial chromosome in the center cell. The phage genome contains the early-acting tsφ1131 gene which codes for the intracellular protein responsible for conferring competence.
PROPHAGE
STATE

HELPER-PHAGE

SUPER-INFECTION
ENHANCEMENT

INTRACELLULAR
EARLY-GENE
PRODUCT
Finally, protein synthesis was shown to be necessary for the expression of competence in \( \phi 11 \)-lysogens, and the growth medium from 8325-4(\( \phi 11 \)) could be used to confer competence to 8325-4. Treatment of the 8325-4 growth medium with Pronase (100 \( \mu \)g/ml) for 15 min before exposure to the 8325-4 cells resulted in a reduction of the competence-conferring ability of that medium (Sjöström et al., 1973). The ability of the growth medium from the \( \phi 11 \)-lysogen to confer competence to a noncompetent organism was attributed by the authors to free \( \phi 11 \) particles present in the medium that infected and lysogenized the 8325-4 cells. The observation that this competence-conferring ability of the medium could be reduced by treating the medium with Pronase was not explained.

In order to determine the role that \( \phi 11 \) plays in conferring competence to 8325, Sjöström and Philipson (1974) isolated a number of temperature-sensitive mutants of \( \phi 11 \) (\( ts\phi 11 \)). These mutants were used to infect 8325-4, and the isolated lysogens were tested for the ability to undergo transfection and transformation at the nonpermissive temperature. One of these mutants (\( ts\phi 11_{31} \)) was unable to confer competence as a prophage at the nonpermissive temperature. This mutant was then used in genetic crosses with a variety of suppressor-sensitive mutants of \( \phi 11 \) isolated and characterized by Kretschmer and Egan (1973; 1975). The \( ts\phi 11_{31} \) mutation mapped in the region that Kretschmer and Egan (1975) had designated to be the early region of the \( \phi 11 \) genome. Therefore, Sjöström and Philipson (1974) concluded that the competence-conferring ability of \( \phi 11 \) is due to the intracellular product of the early phage gene. They hypothesized that the phage confers competence
in the prophage state by an occasional derepression of the early gene. In the vegetative state it functions as a helper-phage by a transient expression of the early gene before the phage genome is suppressed and integrates into the host chromosome. In a superinfecting capacity, the superinfecting phage increases the level of competence in the cell by duplication of the early gene (Fig. 1).

It was the purpose of this study to examine the validity of the model proposed by Sjöström and Philipson (1974) and to investigate more thoroughly the involvement of phage in the expression of competence in *S. aureus*. A preliminary report of some of the findings presented in this dissertation has already appeared (Thompson and Pattee, 1977).
MATERIALS AND METHODS

Bacterial Strains and Bacteriophages

Derivatives of *Staphylococcus aureus* strain 8325 that were used in this study are listed in Table 2 along with their origins and descriptions. The propagating strains for the *Staphylococcus* reference typing phages were obtained from the Center for Disease Control (CDC), Atlanta, GA. These strains are designated according to the phage that is customarily propagated on that strain. For example, Ps55 is the propagating strain (Ps) for typing phage 55. Novobiocin-resistant transductants of the propagating strains were obtained after infection with either phage 80 propagated on U9bla tet ol nov-142 (Pattee and Baldwin, 1962) or phage 83A propagated on 655nov-142 (Pattee et al., 1968b). Thymine-dependent mutants of the propagating strains were obtained by incubation of the propagating strain in the presence of trimethoprim (see below). Genetically marked derivatives of the propagating strains are listed in Table 3. All cultures were maintained on brain heart infusion (BHI; Difco, Detroit, MI) agar slants supplemented with thymine (20 µg/ml) and adenine, guanine, cytosine and uracil (5 µg each/ml) and stored at 4°C.

The *Staphylococcus* reference typing phages were obtained from CDC and propagated on their respective propagating strains. Phage 80α was obtained from F. E. Young (University of Rochester, Rochester, NY) and has been described (Novick, 1963). Phages φ11 and φ12 were isolated from a single plaque from strains 8325-4(φ11) and 8325-4(φ12), respectively; these phages have also been described (Novick, 1967). Phages 80α, φ11
<table>
<thead>
<tr>
<th>Designation</th>
<th>Genotype</th>
<th>Origin</th>
<th>Description and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP1</td>
<td>8325 thy-101</td>
<td>This lab</td>
<td>Single clone from ISP18 (Pattee and Neveln, 1975)</td>
</tr>
<tr>
<td>ISP2</td>
<td>8325 nov-142</td>
<td>This lab</td>
<td>ISP17 after transformation with ISP11 DNA (Pattee and Neveln, 1975)</td>
</tr>
<tr>
<td>ISP4</td>
<td>8325 thy-101 thrB106</td>
<td>This lab</td>
<td>ISP1 after nitrosoguanidine mutagenesis (Pattee and Neveln, 1975)</td>
</tr>
<tr>
<td>ISP5</td>
<td>8325 thy-101 thrB106 ilv-129</td>
<td>This lab</td>
<td>ISP4 after nitrosoguanidine mutagenesis (Pattee and Neveln, 1975)</td>
</tr>
<tr>
<td>ISP8</td>
<td>8325-4</td>
<td>F. E. Young^a</td>
<td>Wild-type 8325 cured of prophages φ11, φ12 and φ13 (Novick, 1967)</td>
</tr>
<tr>
<td>ISP9</td>
<td>8325-4(φ12)</td>
<td>R. P. Novick^b</td>
<td>ISP8 lysogenized with phage φ12 (Novick, 1967)</td>
</tr>
<tr>
<td>ISP10</td>
<td>8325-4(φ13)</td>
<td>R. P. Novick^b</td>
<td>ISP8 lysogenized with phage φ13 (Novick, 1967)</td>
</tr>
<tr>
<td>ISP11</td>
<td>8325(pI258)</td>
<td>M. Lindberg^c</td>
<td>Strain 8325 carrying the penicillinase plasmid pI258 (Novick, 1967)</td>
</tr>
<tr>
<td>ISP17</td>
<td>8325 thy-101 nov-142</td>
<td>This lab</td>
<td>ISP1 after transformation with DNA from strain 655 nov-142 (Pattee and Neveln, 1975)</td>
</tr>
<tr>
<td>ISP18</td>
<td>8325 thy^-</td>
<td>M. Lindberg^c</td>
<td>Thymine-dependent mutant of strain 8325 (Lindberg et al., 1972)</td>
</tr>
<tr>
<td>ISP89</td>
<td>8325-4(φ11)</td>
<td>R. P. Novick</td>
<td>ISP8 lysogenized with phage φ11 (Novick, 1967)</td>
</tr>
</tbody>
</table>
This lab

ISP5 after transformation with DNA strain Ps6 tmn-3110

^Frank E. Young, University of Rochester, Rochester, NY.


^Martin Lindberg, University of Uppsala, Uppsala, Sweden.

^Described in Pattee et al. (1968b).

^83A^ designates the absence of all or part of the φ11 prophage; φ11 and 83A show cross immunity (Sjöström and Philipson, 1974). pig^t^ denotes the production of pigment which all other strains used in this study do not demonstrate (because they possess the pig-131 marker).

^Described by Asheshov (1975); obtained from E. H. Asheshov (Central Public Health Laboratory, London, England) and also described in Pattee (1976).
Table 3. Designations, genotypes, and origins of the genetically-marked derivatives of the propagating strains (Ps) of the Staphylococcal reference typing phages.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP133</td>
<td>Ps 3A <em>thv-133</em></td>
<td>Ps 3A after trimethoprim selection</td>
</tr>
<tr>
<td>ISP134</td>
<td>Ps 3C <em>thy-134</em></td>
<td>Ps 3C after trimethoprim selection</td>
</tr>
<tr>
<td>LSP135</td>
<td>Ps 55 <em>thy-135</em></td>
<td>Ps 55 after trimethoprim selection</td>
</tr>
<tr>
<td>ISP136</td>
<td>Ps 71 <em>thy-136</em></td>
<td>Ps 71 after trimethoprim selection</td>
</tr>
<tr>
<td>ISP137</td>
<td>Ps 42D <em>thy-137</em></td>
<td>Ps 42D after trimethoprim selection</td>
</tr>
<tr>
<td>ISP138</td>
<td>Ps 42E <em>thy-138</em></td>
<td>Ps 42E after trimethoprim selection</td>
</tr>
<tr>
<td>ISP139</td>
<td>Ps 187 <em>thy-139</em></td>
<td>Ps 187 after trimethoprim selection</td>
</tr>
<tr>
<td>ISP140</td>
<td>Ps 29 <em>nov-142</em></td>
<td>Ps 29 after transduction with 80/ ISP217a</td>
</tr>
<tr>
<td>ISP141</td>
<td>Ps 52 <em>nov-142</em></td>
<td>Ps 52 after transduction with 80/ ISP217</td>
</tr>
<tr>
<td>ISP142</td>
<td>Ps 52A/79 <em>nov-142</em></td>
<td>Ps 52A/79 after transduction with 80/ ISP217</td>
</tr>
<tr>
<td>ISP143</td>
<td>Ps 80 <em>nov-142</em></td>
<td>Ps 80 after transduction with 80/ ISP217</td>
</tr>
<tr>
<td>ISP144</td>
<td>Ps 81 <em>nov-142</em></td>
<td>Ps 81 after transduction with 80/ AIP217</td>
</tr>
<tr>
<td>ISP145</td>
<td>Ps 6 <em>nov-142</em></td>
<td>Ps 6 after transduction with 83/ ISP211b</td>
</tr>
<tr>
<td>ISP146</td>
<td>Ps 53 <em>nov-142</em></td>
<td>Ps 53 after transduction with 83/ ISP211</td>
</tr>
<tr>
<td>ISP147</td>
<td>Ps 54 <em>nov-142</em></td>
<td>Ps 54 after transduction with 83/ ISP211</td>
</tr>
<tr>
<td>ISP148</td>
<td>Ps 75 <em>nov-142</em></td>
<td>Ps 75 after transduction with 80/ ISP217</td>
</tr>
<tr>
<td>ISP149</td>
<td>Ps 77 <em>nov-142</em></td>
<td>Ps 77 after transduction with 83/ ISP211</td>
</tr>
<tr>
<td>ISP150</td>
<td>Ps 83A <em>nov-142</em></td>
<td>Ps 83A after transduction with 83/ ISP211</td>
</tr>
</tbody>
</table>
ISP151  Ps 84 nov-142  Ps 84 after transduction with 80/ ISP217
ISP152  Ps 85 nov-142  Ps 85 after transduction with 83/ ISP211

^aPhage 80 propagated on strain U9bla tet ol nov-142 (ISP217), described by Pattee and Baldwin (1962).

^bPhage 83A propagated on strain 655 nov-142 (ISP211), described by Pattee et al. (1968b).
and φ12 were propagated on strain 8325-4. All phages were maintained in trypticase soy broth (TSB; BBL, Cockeysville, MD) or suspension medium and stored at 4°C.

Culture Media

All phages were propagated and assayed on trypticase soy agar (TSA; BBL) supplemented with $5 \times 10^{-3}$ M $\text{CaCl}_2$ by using a soft-agar overlay of trypticase soy broth (TSB; BBL) plus 0.5% (w/v) agar (Difco); these media were also used to assay for transfectants. A complete defined synthetic (CDS) medium has been described for the selection of auxotrophic markers in *S. aureus* (Pattee and Neveln, 1975). This medium consists primarily of a phosphate-buffered glucose-salts solution containing the 18 natural amino acids and several essential vitamins. Thymine-independent (Thy$^+$) transformants were recovered on CDS agar devoid of thymine, and Thy$^+$Thr$^+$ cotransformants were recovered on CDS agar devoid of thymine and threonine. Novobiocin-resistant transductants and transformants were recovered on BHI agar containing 10 μg of novobiocin per ml and 0.05% sodium citrate. Selection for Thy$^+$Nov$^r$ cotransformants was on CDS devoid of thymine and containing 10 μg of novobiocin per ml; single selection for Nov$^r$ in this experiment was on CDS agar containing 10 μg of novobiocin per ml. Viable cell counts were determined on BHI agar. Cells to be transformed or transfected were always grown in TSB. All commercially available dehydrated media were prepared according to the manufacturer's specifications and supplemented with 20 μg thymine per ml when required.
Reagents and Buffers

The following reagents were used frequently in this study; therefore, they were prepared in large volumes and frozen (-45 C) in convenient aliquots. Pronase stock (10,000 µg/ml) was prepared by dissolving crystalline Pronase (Calbiochem, San Diego, CA) in 0.85% NaCl, passing it through a sterile 02 Selas filter (Selas Flotronics, Huntingdon Valley, PA), and incubating it at 35 C for 30 min to destroy any DNase activity. Lysostaphin stock (1 mg/ml) was prepared by dissolving Lysostaphin (Swartz-Mann, Orangeburg, NY) in Tris-NaCl buffer (600 mg Tris(hydroxymethyl)aminomethane, 870 mg NaCl, 100 ml deionized H₂O; pH 7.5). Deoxyribonuclease I (DNase I; 1 mg/ml; Sigma) was dissolved in 0.005 M MgSO₄. Novobiocin (10,000 µg/ml; Sigma) was dissolved in deionized H₂O and filter sterilized.

The following buffers were used frequently in this study; therefore, they were prepared in large volumes and held at room temperature. Borate-buffered saline consisted of 1.5 g boric acid, 2.4 g sodium tetraborate, 250 ml deionized H₂O; this was diluted 1:20 with 0.85% NaCl and the pH was adjusted to 8.5 (Campbell et al., 1970). Saline-sodium citrate (SSC) contained 0.015 M trisodium citrate in 0.15 M NaCl (Marmur, 1961) at pH 7.2. Tris-maleate contained 0.1 M Tris(hydroxymethyl)aminomethane and 0.03 M maleic anhydride at pH 7.0 (Lindberg et al., 1972). Phage suspension medium was prepared as a 10X stock and diluted before use; the stock contained 6 ml of 1 M Tris (pH 7.5), 0.12 g MgSO₄, 4 g NaCl, 0.05 g gelatin, and 1000 ml H₂O (Weigle et al., 1959).
Propagation of Bacteriophages

Overnight BHI agar slant cultures of the propagating strain were suspended in 1.0 ml saline (0.85% NaCl); 0.2 ml of this suspension (approximately $2 \times 10^9$ cells) was added to 4.0 ml of liquified, tempered (45 C) soft-agar. Enough phage lysate was added to achieve a multiplicity of infection (MOI) of approximately 5 (0.1 ml of a lysate with a titer of $1 \times 10^{10}$ plaque-forming-units per ml). The soft-agar was gently mixed and poured onto prepoured TSA. The plates were incubated upright at 35 C for 6 h. The soft-agar was then homogenized in 10 ml of either TSB or phage suspension medium. Cells and cell debris were removed by centrifugation (3,400 x g, 10 min, room temperature), and the supernatant (phage lysate) was passed through a sterile 02 Selas filter. The filtrate was transferred to a sterile culture tube and stored at 4 C. Sterility of the lysate was confirmed by inoculating the surface of a BHI agar plate with 0.1 ml of the lysate and incubating it at 35 C for 24 h.

For propagation of a large volume of lysate, the above procedure was scaled up by use of Pyrex baking dishes (26.5 x 17 cm). The area of one baking dish was approximately equal to the combined areas of 12 standard (100 mm diameter) Petri dishes. Thus the yield from one baking dish was approximately 120-150 ml of lysate. Sterility was maintained by the use of covers constructed from aluminum foil.

The titer of each phage lysate was determined by inoculating 4.0 ml of liquified, tempered (45 C) soft-agar with approximately $1 \times 10^7$ cells per ml of the propagating strain that had been harvested from a
12-h BHI agar slant culture. Serial 10-fold dilutions of the phage lysate were made into TSB, and 0.1 ml of the appropriate dilutions was added in duplicate to soft-agar containing the indicator cells. The soft-agar was then gently mixed and poured onto predried TSA. The plates were incubated at 35 C for 12 h, plaques were counted, and titers were expressed in plaque-forming-units (pfu) per ml.

Purification of Bacteriophages

Approximately 1200 ml of crude lysate was centrifuged at 48,700 x g for 2 h at 20 C; this procedure was found to remove 99.9% of the infectious phage particles from the crude lysate. Each pellet was overlaid with 0.5 ml of suspension medium and allowed to resuspend overnight at 4 C. The pellets were pooled and emulsified by pipetting them through a 10 ml pipette with a broken tip. Residual agar was removed by low speed centrifugation (3,400 x g, 10 min, room temperature). Remaining phage were recovered from the agar by resuspending the pellet in a few ml of suspension medium, emulsifying with a pipette, and removing the agar by centrifugation; this procedure was repeated until the supernatant no longer appeared smoky-blue (indicative of concentrated phage particles). The supernatants from the low speed centrifugations were pooled, and 45% (w/w) technical grade CsCl (Apache Chemicals, Seward, IL) was added and allowed to dissolve at room temperature. The actual density of the solution was adjusted to 1.50 g/cm$^3$ using refractometry (Abbe-3L Refractometer, Bauch and Lamb, Rochester, NY). This phage-CsCl mixture was centrifuged to equilibrium (61,000 x g, at least 20 h, 20 C) in 4-ml
polyallomer centrifuge tubes. The phage equilibrated at approximately the middle of this isopycnic gradient and were discernible as a smoky-blue band. The phage were removed by puncturing the tubes from the side with a 26 gauge needle and slowly extracting the phage into a syringe. The CsCl was removed by dialysis at 4 C against successive 12-h changes (1000 ml each) of suspension medium containing decreasing concentrations (2.5, 2.0, 1.5, 1.0, 0.5, and 0.2 M) of NaCl.

Isolation of Deoxyribonucleic Acid (DNA)

**Bacterial DNA**

Bacterial DNA for transformations was prepared according to the procedure of Lindberg et al. (1972) as modified by Pattee and Neveln (1975). Cells of the culture from which DNA was to be isolated were inoculated into 150-ml volumes of BHI and incubated at 35 C with aeration until stationary phase was reached (usually overnight). The cells were harvested by centrifugation (10,000 x g, 30 min, 4 C), washed once with Tris-NaCl EDTA buffer [0.1 M Tris(hydroxymethyl)aminomethane, 0.1 M NaCl, 0.1 M (ethylenedinitrilo)-tetraacetic acid, pH 7.4], resuspended in 5 ml of the same buffer, and transferred to a 50-ml screw-capped Erlenmeyer flask. Lysostaphin was added to achieve a final concentration of 300 µg/ml, and the mixture was incubated at 35 C until lysis occurred (generally about 0.5 h). Pronase was added to achieve a final concentration of 10 mg/ml. The Pronase was thoroughly mixed with the lysate by gently shaking for 5 min, after which the lysate was allowed to sit for 55 min at 35 C without
agitation. Approximately 0.5 ml of sodium dodecyl sulfate (5% SDS in 45% ethanol) was added to the lysate, and the mixture was shaken vigorously for 30 min on a Burrell wrist-action shaker (Burrell Corp., Pittsburgh, PA). An equal volume (about 5 ml) of phenol that had been freshly distilled (under N₂) and saturated with 0.01 M Tris-HCl buffer (pH 8.1) was added, and the mixture was roller-extracted, using a Kraft RD-30-18 rotor head (Kraft Apparatus Inc., Mineola, NY) rotating on a horizontal axis at 60 rpm for 30 min under reduced illumination. The emulsion was broken by centrifugation (8,000 × g, 45 min, 4°C). The DNA-containing aqueous layer was carefully removed, and the phenol extraction and centrifugation were repeated. Residual phenol left after the second de-proteinization was extracted with an equal volume of ethyl ether. Residual ether was removed by gently blowing N₂ gas into the preparation. The DNA was precipitated from the solution by overlaying it with 2 volumes of cold (4°C) 95% ethanol. The resulting DNA fibers were gently spooled onto a prescored glass rod, and the precipitated DNA was held at 4°C in 95% ethanol for at least 12 h. The glass rod was broken along the score, and the DNA was allowed to dissolve in 4 ml of SSC. The sterility of the DNA was confirmed by inoculating 0.1 ml onto the surface of a predried BHI agar plate and incubating at 35°C for 24 h. The DNA was assayed by using the diphenylamine colorimetric assay (Burton, 1956).

Bacteriophage DNA

Bacteriophage DNA for transfections was prepared according to a modification of the procedure of Sjostrom et al. (1972). DNA was
released from purified 80α-phage particles (approximately $5 \times 10^{13}$) by the addition of sodium dodecyl sulfate (0.5% final concentration) and incubation at 35°C for 30 min. The DNA was then either extracted with phenol before precipitation as described for the bacterial DNA or precipitated from solution with cold (4°C) 95% ethanol without the phenol extraction steps. Before use, the DNA was dissolved in approximately 10-15 ml of SSC, tested for sterility on BHI agar, and quantitatively assayed by using the diphenylamine assay.

Both of these procedures yielded DNA preparations that were equally infective when assayed by transfection; in addition, the transfecting activity of both was completely destroyed by treatment with DNase I (0.1 mg/ml, 10 min, room temperature), and neither preparation was infective without the presence of competent cells and 0.1 M CaCl$_2$ (see below). However, the phenol-extracted DNA proved to be more stable during long-term storage than the DNA that had not been deproteinized with phenol.

**Transformation Procedure**

The preparation of competent cells of strain 8325 for transformation and the transformation procedure were according to Lindberg et al. (1972) as modified by Pattee and Neveln (1975). Cells from an overnight BHI agar slant culture (18 x 150 mm) were suspended in 5 ml saline (0.85% NaCl); a sufficient volume (about 1 ml) of this cell suspension was added to 100 ml of TSB in a 300-ml nephelometer flask to attain an optical density (O.D.) of 0.01 ($\lambda = 540$ nm). Ten-fold dilutions of this cell suspension were made into 100-ml volumes of TSB contained in 300-ml
nephelometer flasks (to attain an O.D. of 0.01), and the cells were incubated with reciprocal shaking (100 cycles per min, 3.8 cm strokes) at 35 C until the absorbancy reached 0.10. The cells were harvested by centrifugation (10,000 x g, 30 min, 4 C), washed once with cold (4 C) Tris-maleate buffer, and resuspended in 1.0 ml of the same buffer (per 100 ml of original culture). Eight-tenths milliliter of the cell suspension was added to 0.1 ml of a saturating concentration of DNA; 0.1 ml of DNA pretreated with DNase I (0.1 mg/ml, 10 min, room temperature) and 0.1 ml of SSC served as control transformation mixtures. Then 0.1 ml of 1 M CaCl$_2$ was added to the transformation mixtures. The cells were incubated in the presence of DNA and CaCl$_2$ at room temperature for 5 min. The cells were harvested by centrifugation (3,400 x g, 7 min, room temperature), resuspended in BHI and incubated at 35 C for 30 min to allow for phenotypic expression and segregation of markers. The cells were again centrifuged (3,400 x g, 7 min, room temperature), resuspended in 1.0 ml of saline, and 0.1 ml of the cell was surface inoculated onto selective media. Dilutions of the transformed cells were made into saline and plated onto BHI agar to determine viable cell counts. All plates were incubated at 35 C and scored after 24-48 h, depending upon the rate of growth of the culture.

Transfection Procedure

The preparation of competent cells of strain 8325 for transfection and the transfection procedure were similar to those described above for transformation (Sjostrom et al., 1973) with the following modifications.
A sufficient volume of the cells suspended from the BHI agar slant was added to 100 ml of TSB to attain an initial O.D. (540) of 0.50. Ten-fold dilutions of this cell suspension were then made into 100-ml volumes of TSB (to attain an O.D. of 0.05), and the cells were incubated with shaking until the O.D. reached 0.20. The cells were harvested, washed in Tris-maleate buffer and resuspended in 2 ml of the same buffer (per 100 ml of the original culture). Eight-tenths milliliter of cells was added to 0.1 ml of a saturating concentration of 80α DNA or DNA pretreated with DNase (0.1 mg/ml, 10 min, room temperature); then 0.1 ml of 1 M CaCl₂ was added, and the cells were allowed to incubate in the presence of DNA and CaCl₂ for 5 min at room temperature. The cells were centrifuged (3,400 X g, 7 min, room temperature), resuspended in 1.0 ml TSB, and incubated with shaking at 35 C for 10 min. Transfectants were assayed by making appropriate 10-fold dilutions into saline, adding 0.1-ml samples of the dilutions to soft-agar (4.0 ml) containing cells of the appropriate indicator strain, and pouring the soft-agar onto predried TSA plates. Viable cell counts were also determined for each sample by surface inoculating predried BHI agar plates with 0.1 ml of the appropriate cell dilutions. The plates were incubated at 35 C for at least 12 h. A slight modification of this procedure was used in certain experiments to accommodate smaller sample volumes.

Transduction Procedure

The procedure for the transduction of genetic markers was essentially that of Kasatiya and Baldwin (1967). The generalized transducing phages 80 and 83A were propagated on the appropriate donor strain
according to the procedure described previously. Overnight BHI agar slant cultures (18 x 150 mm) of the recipient strains were suspended in 1.0 ml of TSB which had been supplemented with $5 \times 10^{-3}$ M CaCl$_2$. A 0.5-ml aliquot of the suspended cells was transferred to a centrifuge tube containing 1.5 ml of TSB + $5 \times 10^{-3}$ M CaCl$_2$. A sufficient volume of the transducing phage lysate was added to achieve an MOI of about 1; the control transduction suspensions received an equal volume of TSB. The cells were incubated with the phage at 35 C with shaking (100 cycles/min, 3.8 cm strokes) for 30 min. The infection was stopped by the addition of 1.0 ml of cold (4 C) sodium citrate (0.02 M, pH 7.0). The cells were removed by centrifugation (3,400 x g, 7 min, room temperature), resuspended in cold (4 C) sodium citrate, and 0.1-ml volumes were surface-inoculated onto BHI agar containing 10 µg of novobiocin per ml and 0.05% sodium citrate. The plates were incubated at 35 C for 24-48 h, and the resulting transductants were purified by repeated streaking onto BHI agar containing 10 µg of novobiocin per ml.

Mutagenesis Procedure

Thymine-dependent mutants were obtained by a modification of the procedure of Okada et al. (1960). Cells from an overnight BHI agar slant culture were used to inoculate a 4-ml volume of CDS broth; this culture was incubated without shaking at 35 C for 12 h at which time stationary phase had been attained. A 10-fold dilution of the CDS broth culture was made into saline and 0.1 ml volumes of this dilution were used to inoculate CDS broth containing 12 µg of thymidine and 10-30 µg of filter-
sterilized trimethoprim (Burroughs Wellcome and Company, Inc., Research Triangle Park, NC) per ml. Control cultures contained thymidine but were devoid of trimethoprim. These cultures were incubated at 35 °C under reduced illumination for 2 days. Thymine-dependent mutants were isolated by streaking the CDS broth cultures onto BHI agar containing 10 µg each of thymine and thymidine per ml. The thymine-dependent nature of the mutants was confirmed by auxanography on CDS agar devoid of thymine and thymidine.

Sera

Preparation of antisera

Phages φ11 and φ12 were purified as described above except that they were dialyzed against decreasing concentrations of NaCl in suspension medium devoid of gelatin. Approximately 5 x 10^9 pfu were emulsified with an equal volume of Freund's complete adjuvant (Difco) for each injection. Five injections were given intramuscularly to rabbits at 4-day intervals, and the animals were exsanguinated 7 days after the final injection. The blood was allowed to clot at room temperature, and the serum was aseptically removed and stored at -45 °C.

Normal rabbit serum

Normal rabbit serum was obtained commercially (Granite Diagnostics, Inc., Burlington, NC) or by cardiac puncture of rabbits before the injections were commenced. Normal serum was stored at -45 °C.
Precipitation of gamma-globulins

The gamma-globulin-containing fraction of antiserum and normal serum was precipitated from the whole sera by using the ammonium sulfate precipitation procedure of Campbell et al. (1970). A saturated solution of ammonium sulfate (pH 7.8) was added slowly with constant stirring to 10 ml of serum until a final volume of 15 ml was attained (thus effecting one-third saturation). The suspension was allowed to stir at room temperature for 2 h to avoid mechanical occlusion of undesirable serum components. The precipitate was removed by centrifugation (3,400 x g, 15 min, room temperature) and dissolved in 10 ml of borate-buffered saline. This procedure was repeated twice, and the final precipitate was dissolved in 3 ml of borate-buffered saline. The fraction was dialyzed against six 12-h changes of borate-buffered saline (500 ml each) at 4 C.

Antiserum neutralization test

Antisera were assayed for the ability to neutralize infectious phage particles by using the constant-time, variable-antibody concentration procedure of Eisenstark (1967). A crude phage lysate was diluted into TSB to attain a titer of 5 x 10^7 pfu per ml. The homologous antiserum was diluted 1:10, 1:100, and 1:1000 with TSB. The diluted phage and the antiserum dilutions were preincubated at 35 C for 10 min, then 0.5 ml of the phage was mixed with 0.5 ml of each antiserum dilution. The neutralization mixtures were incubated at 35 C for 10 min after which each was quickly diluted 1:100 with TSB. One-tenth ml of each dilution was added in duplicate to 4-ml volumes of soft-agar containing the appropriate
indicator cells, and the soft-agar was poured onto predried TSA plates. The antiserum dilution that yielded 50–250 plaques per plate was used to determine the $k$-value according to the following equation:

$$k = 2.3 \times \frac{D}{t} \times \log \left( \frac{P_0}{P} \right)$$

where $D$ is the final dilution of antiserum, $t$ is the time in minutes, $P_0$ is the initial population of infectious particles, and $P$ is the population of infectious particles after antiserum treatment.

**Bacteriophage Typing**

The phage typing patterns of the strains used in this study were determined by using the procedures described by CDC (1969) except that the typing was performed on TSA containing $5 \times 10^{-3}$ M CaCl$_2$.

**Sonication of Cells**

Cells that had been exposed to serum during the transformation or transfection process had a tendency to aggregate. Therefore, before plating it was necessary to disrupt the aggregates by a brief sonication which does not appear to affect cell viability (R. P. Novick, personal communication). Sonication was performed in an ice-bath at 50% output of a Biosonik II (Bronwill Scientific, Rochester, NY) sonicator equipped with a needle probe for 15 sec per ml of sample.
RESULTS

Preliminary Observations

The original question addressed by this study concerned the expression of competence in *Staphylococcus aureus* strain 8325 as it might be related to the natural division cycle of the bacterium. Because the experiments designed to investigate this question resulted in a major shift in the direction taken by the research, the question remains unanswered. However, these experiments provided a great deal of insight into the nature of the competent state in *S. aureus* strain 8325. Therefore a brief synopsis of the results of some of these experiments is presented here.

The strategy employed to investigate the expression of competence as it might be related to the natural division cycle of the bacterium involved using a method by which a population of cells might be synchronized without disrupting the metabolic integrity of the cells. The method used had been described by Chatterjee et al. (1971) using *S. aureus* H. These investigators found that a synchronously dividing culture could be obtained by separating cells on the basis of sedimentation rates in a continuous sucrose gradient; the slowly sedimenting cells were used to initiate a synchronously growing culture. However, they found that satisfactory synchrony was obtained only if the slowly sedimenting cells were reinoculated into the medium in which the cells had previously grown to early log phase (so-called "conditioned" medium).
In preliminary experiments with *S. aureus* 8325nov-142, a Nov\(^R\) transductant of the wild-type organism that carries the prophages \(\phi 11\), \(\phi 12\), and \(\phi 13\), some semblance of synchrony (approximately 50% of the cells dividing synchronously) was observed if the slowly sedimenting cells were reinoculated into the filtered medium in which they had previously grown to early log phase. The low cell density and the low number of synchronous divisions obtained by this method proved to be detrimental to examining physiological differences that might occur during the natural growth cycle. However, samples taken at intervals during the first complete division cycle and transfected with 80\(\alpha\) DNA showed a reproducible increase in the level of competence at the time of septum formation.

There appeared to be four plausible explanations to account for this increase. The increase in the level of competence might be due to (1) a dose-response resulting from the replication of the chromosome and thus the duplication of the early phage gene proposed by Sojström and Philipson (1974), (2) a general nonspecific increase in permeability of the cell during septum formation, (3) a doubling of the DNA receptor sites on the cell surface at the time of division, (4) a competence factor that is produced or functions most efficiently at the time of cell division. Of these four possibilities, the fourth appeared to be the most easily tested.

A culture of 8325nov-142 that had been grown to early log phase was separated on a sucrose gradient, and the slowly sedimenting cells were used to inoculate filtered growth medium in which 8325-4, a derivative of
8325 that has been cured of the prophages \(\phi11\), \(\phi12\), and \(\phi13\) and cannot become naturally competent, had grown to early log phase. In this "conditioned" medium the 8325nov-142 cells did not exhibit an increase in the expression of competence at the time of cell division.

Sjöström and Philipson (1974) had reported that the only extracellular factor that could be transferred from competent to noncompetent cells in *S. aureus* 8325 was infectious \(\phi11\) particles. However, the discrepancy in the pattern of competence observed in the "conditioned" medium from competent cells and that observed in the "conditioned" medium from noncompetent cells indicated that an extracellular factor, perhaps free \(\phi11\), did play a role in the acquisition of competence in strain 8325. Therefore, the growth medium from competent cells was examined.

**Transfer of Competence with Growth Media**

Comparison of growth media from 8325nov-142 and 8325-4(\(\phi11\))

Sjöström et al. (1973) reported that a culture medium in which 8325-4(\(\phi11\)) had grown to competence contained between \(1 \times 10^4\) and \(1 \times 10^6\) pfu/ml and conferred competence to 8325-4. They attributed this acquisition of competence to free \(\phi11\) particles present in the culture medium (Sjöström et al., 1973; Sjöström and Philipson, 1974) that lysogenized the noncompetent cells (Rudin et al., 1974).

In this study, growth media from competent 8325-4(\(\phi11\)) and competent 8325nov-142 were examined for the ability to confer competence to 8325-4(\(\phi12\)), another derivative of 8325 that normally cannot become competent. Cultures of 8325-4(\(\phi11\)), 8325nov-142, and 8325-4(\(\phi12\)) were used
to inoculate 100-ml volumes of TSB. Cells were grown to early log
(O.D. = 0.20), harvested, and assayed for competence for transfection
with 80α DNA. The culture media from 8325-4(φ11) and 8325nov-142 were
passed through 0.45 μm membrane filters (Millipore, type HAWP) and then
equilibrated at 35 C. Each medium was inoculated with cells from 100 ml
of TSB culture of 8325-4(φ12); the cells were incubated with shaking for
20 min, harvested, and assayed for competence by transfection with 80α
DNA. Table 4 shows that after 20 min of exposure to 8325nov-142 growth
medium, 8325-4(φ12) attained a level of competence almost equal to that
attained in the 8325-4(φ11) growth medium.

Free phage content of media

When the culture media from competent 8325-4 (φ11) and 8325nov-142
were analyzed for their content of free infectious phage particles using
8325-4 as the indicator cells, they were found to contain 2.0 x 10^5
pfu/ml and 4.0 x 10^4 pfu/ml, respectively. Whereas the free infectious
phage particles in the 8325-4(φ11) growth medium could be neutralized
by preincubation with 10% antiserum directed against the purified φ11
at 35 C for 30 min, the free phage content of the 8325nov-142 growth
medium could not be neutralized with this antiserum (data not shown).
However, when the 8325nov-142 growth medium was treated with antiserum
(10%) directed against purified φ12 at 35 C for 30 min and assayed for
lytic activity against 8325-4, only 10 pfu/ml could be detected (Table
5). Treatment of the medium with both φ11- and φ12-antiserum (10% each)
at 35C for 30 min neutralized all lytic activity toward 8325-4. Assays
Table 4. The ability of filtered growth media from competent 8325nov-142 and 8325-4(φ11) to confer competence for transfection to 8325-4 (φ12)

<table>
<thead>
<tr>
<th>Recipient Cells</th>
<th>Treatment</th>
<th>Transfection Frequency&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8325nov-142</td>
<td>None&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 x 10^-4</td>
</tr>
<tr>
<td>8325-4(φ12)</td>
<td>None</td>
<td>&lt;6.0 x 10^-9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8325-4(φ12)</td>
<td>Incubated in filtered growth medium from competent 8325nov-142</td>
<td>1.1 x 10^-5</td>
</tr>
<tr>
<td>8325-4(φ11)</td>
<td>None</td>
<td>1.3 x 10^-5</td>
</tr>
<tr>
<td>8325-4(φ12)</td>
<td>Incubated in filtered growth medium from competent 8325-4(φ11)</td>
<td>1.0 x 10^-5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the number of 80α plaques/number of viable cells exposed to the DNA. Transfection reactions contained a saturating concentration of phage 80α DNA (10 - 15 μg/ml of reaction) and approximately 1 x 10^9 cells/ml of reaction.

<sup>b</sup>Cells collected and assayed for competence.

<sup>c</sup>Less than the given frequency denotes that no transfectants were detected.
Table 5. Analysis of the free phage content of the filtered growth medium from competent 8325nov-142

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Indicator Cells</th>
<th>pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8325-4</td>
<td>$4.0 \times 10^4$</td>
</tr>
<tr>
<td>None</td>
<td>8325-4($\phi 12$)</td>
<td>11</td>
</tr>
<tr>
<td>None</td>
<td>8325-4($\phi 13$)</td>
<td>$3.9 \times 10^4$</td>
</tr>
<tr>
<td>$\phi 12$-antiserum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8325-4</td>
<td>10</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>8325-4</td>
<td>$3.8 \times 10^4$</td>
</tr>
<tr>
<td>$\phi 12$-antiserum and $\phi 11$-antiserum&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8325-4</td>
<td>&lt;1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>$k = 80$

<sup>b</sup>$k = 70$

<sup>c</sup>Denotes no plaques recovered.
for pfu by using 8325-4(\(\phi\)12) and 8325-4(\(\phi\)13) yielded 11 pfu/ml and 1.3 \(\times\) \(10^6\) pfu/ml, respectively. Because \(\phi\)13-lysogens are susceptible to lysis by \(\phi\)12, but \(\phi\)12-lysogens are immune to lysis by both \(\phi\)12 and \(\phi\)13 (Novick, 1967), it appeared that the 8325nov-142 growth medium contained a predominance of infectious \(\phi\)12 particles. Furthermore, \(\phi\)13 is not susceptible to neutralization with antiserum directed against \(\phi\)12 (Rudin and Lindberg, 1975). Therefore, the approximately 10 pfu/ml remaining after neutralization with \(\phi\)12-antiserum or when 8325-4(\(\phi\)12) was used as an indicator were assumed to be either \(\phi\)11 or an unidentifiable serological group B phage.

Neutralization of the competence-conferring activity of the media

Sjöström et al. (1973) demonstrated that the competence-conferring activity of the growth medium from 8325-4(\(\phi\)11) was neutralized by pre-incubation with \(\phi\)11-antiserum, a result that supported their conclusion that free \(\phi\)11 particles are responsible for the competence-conferring activity of the medium. Although there appeared to be an insufficient number of \(\phi\)11 particles in the growth medium from 8325nov-142 (Table 5) to account for the competence-conferring activity observed (Table 4), the effect of \(\phi\)11-antiserum on this activity was determined. The filtered media from both 8325-4(\(\phi\)11) and 8325nov-142 were treated with \(\phi\)11-antiserum (10%) at 35 C for 30 min before 8325-4(\(\phi\)12) cells were added; after incubation for 20 min, the cells were harvested and assayed for competence by transfection with 80\(\alpha\) DNA. The results (Table 6) showed that 97.5% of the competence-conferring activity of the 8325nov-142 growth medium was
Table 6. The ability of filtered growth media from competent 8325nov-142 and 8325-4(φ11) to confer competence for transfection to 8325-4(φ12) after neutralization with φ11-antiserum

<table>
<thead>
<tr>
<th>Source of Medium</th>
<th>Serum</th>
<th>Transfection Frequency$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8325nov-142</td>
<td>Normal</td>
<td>$2.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>8325nov-142</td>
<td>Anti-φ11$^b$</td>
<td>$6.8 \times 10^{-7}$</td>
</tr>
<tr>
<td>8325-4(φ11)</td>
<td>Normal</td>
<td>$1.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>8325-4(φ11)</td>
<td>Anti-φ11$^c$</td>
<td>$&lt; 3.3 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

$^a$Expressed as the number of 80α plaques/number of viable cells exposed to the DNA. Transfection reactions contained a saturating concentrations of phage 80α DNA (10–15 µg/ml of reaction) and approximately 1 x 10^9 cells/ml of reaction.

$^b_k = 70$

$^c$Less than the given frequency denotes that no transfectants were recovered.
neutralized, and no activity was detected with the 8325-4(φ11) growth medium after treatment with φ11-antiserum.

**Assay for lysogeny**

Although the low number of φ11 virions in the growth medium from competent 8325nov-142 (Table 5) did not seem sufficient to account for the competence-conferring activity observed (Table 4), the possibility existed that genetically defective φ11 particles that would not be accounted for by a plaque assay, but that could lysogenize and confer competence by expression of an early gene, might be present in this medium. If lysogeny is a requirement, the recovered transformed cells should exhibit an acquired prophage immunity to φ11. To test this possibility, 8325-4(φ11) and 8325nov-142 filtered growth media were used to confer competence to 8325-4(φ12) as before, but the cells were transformed with DNA isolated from 8325nov-142. From each population of Nov^ transformants, 100 were picked at random and purified by streaking onto BHI agar containing 10 µg novobiocin per ml. These isolates were tested for sensitivity or resistance to φ11 by surface-inoculating a suspension (ca. 1 x 10^8 cfu/ml) of each isolate onto TSA and testing the isolate's response to φ11 at the routine test dilution (RTD; Smith, 1972). All transformants that had been made competent with the 8325nov-142 growth medium remained sensitive to lysis by φ11, and 25 (25%) of the transformants made competent with the 8325-4(φ11) growth medium remained sensitive to lysis by φ11. Thus, the establishment of lysogeny is not a prerequisite (or a consequence) of φ11-confferred competence.
Kinetics of the acquisition of competence

Because 75% of the cells rendered competent by the growth medium from 8325-4(φ11) became lysogenized by φ11, but cells rendered competent by the growth medium from 8325nov-142 showed no indication of lysogeny, it was possible that the two media might confer competence by different mechanisms. Therefore, the kinetics of the acquisition of competence by 8325-4(φ12) cells in these two media were examined. Cultures of 8325-4 (φ11), 8325-4(φ12), and 8325nov-142 were harvested during early log (O.D. = 0.20) and the 8325-4(φ12) cells from 100 ml of culture were suspended in equilibrated (35 C), filtered media from 8325-4(φ11) and 8325nov-142. Samples (5.0 ml) from each medium were removed at intervals, diluted into cold (4 C) Tris-maleate buffer, and assayed for competent cells by transfection with 80ot DNA. Figures 2 and 3 depict the time course of the acquisition of competence by 8325-4(φ12) cells in the growth medium from 8325nov-142 and 8325-4(φ11), respectively. Cells in the 8325nov-142 growth medium reached one distinct level of maximum competence after 20 min of incubation; the cells remained competent for about 100 min, after which competence was rapidly lost. Cells in the 8325-4(φ11) growth medium reached a similar distinct level of competence after about 15-20 min of incubation; however, they also attained a second distinct level of competence after about 60 min of incubation. Although the frequencies fluctuated somewhat after 60 min, no abrupt loss of competence was observed in the 8325-4(φ11) growth medium even after 210 min of incubation. In a separate experiment, 8325-4(φ12) cells were
Fig. 2. Kinetics of the acquisition of competence by 8325-4(φ12) incubated in filtered growth medium from competent 8325nov-142.

Competence was measured by transfection with 80α DNA. Frequency of transfection (open circles) is expressed as the number of 80α plaques/number of viable cells exposed to the DNA (closed circles).
Fig. 3. Kinetics of the acquisition of competence by 8325-4(\phi12) incubated in filtered growth medium from competent 8325-4(\phi11).

Competence was measured by transfection with a saturating concentration of 80\alpha DNA. Frequency of transfection (open circles) is expressed as the number of 80\alpha plaques/number of viable cells exposed to the DNA (closed circles).

Insert: Assay of the 8325-4(\phi11) growth medium for free \phi11 particles during the incubation of 8325-4(\phi12) cells in this medium.
incubated in filtered 8325-4(\(\phi\)11) growth medium as before; samples were removed at intervals, passed through a 0.45 \(\mu\)m filter, and the filtrates were assayed for plaque-forming activity by using 8325-4(\(\phi\)12) as indicator cells. The results (Fig. 3 insert) showed the expected decrease in free \(\phi\)11 as the 8325-4(\(\phi\)12) cells reached the first level of competence. After 40 min the numbers of free \(\phi\)11 particles in the medium were restored but decreased again after 60 min. This pattern suggests a correlation between the restoration of free \(\phi\)11 particles to the medium [resulting from propagation on the 8325-4(\(\phi\)12) cells] and the appearance of the second level of competence in these cells.

Finally, 8325-4(\(\phi\)12) cells that had been rendered competent by incubation in filtered growth media from 8325-4(\(\phi\)11) and 8325nov-142 were tested for the ability to demonstrate competence after being removed from these media. The 8325-4(\(\phi\)12) cells were incubated in the 8325-4(\(\phi\)11) and 8325nov-142 growth media for 20 min, removed by centrifugation, washed once in 0.85% NaCl, and reinoculated into the original filtered (0.45 \(\mu\)m) growth medium from which they had been harvested. Samples (5.0 ml) were removed at intervals and transfected with 80\(\alpha\) DNA as before. The results (Fig. 4) showed that the cells rendered competent by the 8325nov-142 growth medium remained competent for approximately 60 min and then rapidly lost the ability to be transfected. The cells rendered competent by the 8325-4(\(\phi\)11) growth medium not only remained competent for the duration of the experiment (150 min), but they also demonstrated an increase in the frequency of transfection after approximately 80 min.
Fig. 4. Expression of competence by 8325-4(φ12) cells rendered competent by incubation for 20 min in 8325-4(φ11) growth medium (open circles) and 8325nov-142 growth medium (closed circles) and then reinoculation into the original growth medium from which they had been harvested.

Competence was measured by transfection with a saturating concentration of 80α DNA. Frequency of transfection is expressed as the number of 80α plaques/number of viable cells exposed to the DNA.
Survey of phages for the ability to confer competence to 8325-4

The possibility that φ11 confers competence by simply facilitating the uptake of DNA was discounted by Sjöström and Philipson (1974) partially on the basis that superinfection with phages 47 and 75 did not confer competence. Phages 47 and 75, however, are serologically unrelated to phages φ11 and 83A, the two phages that had demonstrated competence-conferring ability (Sjöström and Philipson, 1974). Therefore, various phages (the majority of which are Staphylococcus reference typing phages) were examined for the ability to confer competence to 8325-4.

Preliminary experiments demonstrated that the multiplicity of infection (MOI) might be critical to the recovery of transformed cells. Therefore, the original transformation protocol was modified in the following manner to insure a high MOI in all experiments.

Cultures (100 ml each in TSB) of 8325-4 were harvested during early log growth and suspended in 0.85% NaCl to attain a density of approximately $3 \times 10^9$ cfu/ml; 0.5-ml portions of these cells were added to 15-ml centrifuge tubes containing 1.0-ml volumes of phage lysates (or phage lysates diluted into TSB) that had equilibrated at 35 C. Cells and phages were incubated with gentle shaking for 5 min. The cells were harvested by centrifugation (3,400 x g, 7 min, room temperature), washed once with cold (4 C) Tris-maleate buffer, and resuspended in the same buffer (0.8 ml/tube). Then, to each tube was added either 0.1 ml of a saturating concentration of DNA isolated from 8325nov-142, 0.1 ml of a
saturating concentration of DNA pretreated with DNase I (0.1 mg/ml, 10 min, room temperature), or 0.1 ml of SSC. The addition of the DNA was followed immediately by the addition of 0.1 ml of 1 M CaCl₂. This mixture was incubated at room temperature for 5 min; the cells were removed by centrifugation as before, suspended in BHI (1.0 ml/tube), and incubated at 35 C with shaking for 20 min. The cells were again removed by centrifugation, resuspended in 0.02 M sodium citrate, pH 7.0 (1.0 ml/tube), sonicated for 15 sec/ml, and samples (0.1 ml) were spread onto BHI agar containing 10 μg novobiocin per ml. The number of viable cells in each preparation was also determined, and the transformation frequencies were expressed as the number of transformed cells divided by the number of viable cells exposed to the phage.

Table 7 is a compilation of the abilities of various phage lysates to confer competence to 8325-4. Because these experiments were designed to merely survey a large number of phages for the ability to confer competence, no attempt was made to optimize conditions for each phage tested. The serological group B phages tested were all at least minimally active, and phage 3A, a serological group A phage, also showed limited activity. The competence-conferring activity of phages 55 and 80α was neutralized by preincubation at 35 C for 10 min with 10% φII-antiserum (group B; Novick, 1967); the competence-conferring activity of phage 3A was neutralized by preincubation with 10% φ12-antiserum (serological group A; Rudin and Lindberg, 1975) but not with preincubation with φ11-antiserum (data not shown).
<table>
<thead>
<tr>
<th>Phage</th>
<th>Serological Group(^c)</th>
<th>Lytic Group(^d)</th>
<th>Degree of Lysis at RTD(^e)</th>
<th>MOI(^f)</th>
<th>Transformation Frequency(^b) With 10% Normal Serum</th>
<th>Transformation Frequency(^b) Without Serum</th>
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</thead>
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<tr>
<td>29</td>
<td>B</td>
<td>I</td>
<td>$\n$</td>
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<td>$1.4 \times 10^{-8}$</td>
<td>$7.2 \times 10^{-9}$</td>
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<tr>
<td>52</td>
<td>B</td>
<td>I</td>
<td>$\n$</td>
<td>10</td>
<td>$2.4 \times 10^{-7}$</td>
<td>$1.6 \times 10^{-7}$</td>
</tr>
<tr>
<td>52A</td>
<td>B</td>
<td>I</td>
<td>$\n$</td>
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<td>$3.0 \times 10^{-7}$</td>
<td>$1.8 \times 10^{-7}$</td>
</tr>
<tr>
<td>79</td>
<td>B</td>
<td>I</td>
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<td>$2.0 \times 10^{-7}$</td>
<td>$1.6 \times 10^{-7}$</td>
</tr>
<tr>
<td>80</td>
<td>B</td>
<td>I</td>
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<td>5</td>
<td>$3.4 \times 10^{-7}$</td>
<td>$2.8 \times 10^{-7}$</td>
</tr>
<tr>
<td>55</td>
<td>B</td>
<td>II</td>
<td>$-\n$</td>
<td>10</td>
<td>$2.2 \times 10^{-8}$</td>
<td>$7.7 \times 10^{-7}$</td>
</tr>
<tr>
<td>71</td>
<td>B</td>
<td>II</td>
<td>$-\n$</td>
<td>55</td>
<td>$7.4 \times 10^{-8}$</td>
<td>$7.9 \times 10^{-8}$</td>
</tr>
<tr>
<td>53</td>
<td>B</td>
<td>III</td>
<td>+++</td>
<td>55</td>
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<td>$4.7 \times 10^{-7}$</td>
</tr>
<tr>
<td>83A</td>
<td>B</td>
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<td>+++</td>
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<td>$1.3 \times 10^{-7}$</td>
<td>$1.8 \times 10^{-8}$</td>
</tr>
<tr>
<td>85</td>
<td>B</td>
<td>III</td>
<td>+++</td>
<td>5</td>
<td>$6.0 \times 10^{-7}$</td>
<td>$6.4 \times 10^{-8}$</td>
</tr>
<tr>
<td>95</td>
<td>B</td>
<td>Misc(^g)</td>
<td>Nt(^h)</td>
<td>30</td>
<td>$5.5 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-7}$</td>
</tr>
<tr>
<td>96</td>
<td>B</td>
<td>Misc</td>
<td>Nt(^h)</td>
<td>30</td>
<td>$3.6 \times 10^{-8}$</td>
<td>$6.9 \times 10^{-8}$</td>
</tr>
<tr>
<td>φ11</td>
<td>B</td>
<td>NA(^i)</td>
<td>+++</td>
<td>40</td>
<td>$2.7 \times 10^{-7}$</td>
<td>$1.6 \times 10^{-7}$</td>
</tr>
<tr>
<td>80α</td>
<td>B</td>
<td>NA</td>
<td>+++</td>
<td>60</td>
<td>$1.4 \times 10^{-6}$</td>
<td>$3.2 \times 10^{-7}$</td>
</tr>
<tr>
<td>3A</td>
<td>A</td>
<td>II</td>
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<td>$9.5 \times 10^{-8}$</td>
<td>Nt(^j)</td>
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<td>3C</td>
<td>A</td>
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</tr>
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<td>III</td>
<td>+++</td>
<td>20</td>
<td>$&lt; 4.6 \times 10^{-9}$</td>
<td>Nt(^j)</td>
</tr>
</tbody>
</table>

\(^{a}\) The ability of various staphylococcal phage to confer competence for transformation to 8325-4

\(^{b}\) Transformation Frequency

\(^{c}\) Serological Group

\(^{d}\) Lytic Group

\(^{e}\) Degree of Lysis at RTD

\(^{f}\) MOI

\(^{g}\) Misc

\(^{h}\) Nt

\(^{i}\) NA

\(^{j}\) Nt
<p>| | | | | | |</p>
<table>
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<th></th>
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<td>47</td>
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<td>III</td>
<td>+++</td>
<td>45</td>
<td>$&lt;4.6 \times 10^{-9}$</td>
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<tr>
<td>54</td>
<td>A</td>
<td>III</td>
<td>+++</td>
<td>15</td>
<td>$&lt;4.6 \times 10^{-9}$</td>
</tr>
<tr>
<td>75</td>
<td>A</td>
<td>III</td>
<td>+++</td>
<td>20</td>
<td>$&lt;4.6 \times 10^{-9}$</td>
</tr>
<tr>
<td>42E</td>
<td>A</td>
<td>III</td>
<td>-</td>
<td>45</td>
<td>$&lt;4.6 \times 10^{-9}$</td>
</tr>
<tr>
<td>81</td>
<td>A</td>
<td>Misc</td>
<td>-</td>
<td>5</td>
<td>$&lt;4.6 \times 10^{-9}$</td>
</tr>
<tr>
<td>77</td>
<td>F</td>
<td>III</td>
<td>-</td>
<td>5</td>
<td>$&lt;4.6 \times 10^{-9}$</td>
</tr>
<tr>
<td>42D</td>
<td>F</td>
<td>IV</td>
<td>-</td>
<td>5</td>
<td>$&lt;7.0 \times 10^{-9}$</td>
</tr>
<tr>
<td>187</td>
<td>L</td>
<td>Misc</td>
<td>-</td>
<td>15</td>
<td>$&lt;7.0 \times 10^{-9}$</td>
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</tbody>
</table>

*Competence was measured by transformation with a saturating concentration of 8325*<sup>nov-142</sup> DNA.

*Expressed as the number of Nov<sup>r</sup> transformants/number of viable cells exposed to phage.

*Wentworth (1963); Novick (1967); P. B. Smith, Center for Disease Control, Atlanta, GA, personal communication, 1976.

*Parker (1972).

*Determined on 8325-4 at the routine test dilution (RTD; Smith, 1972).

*Multiplicity of infection (MOI) not intended to reflect the optimum.

*P. B. Smith, personal communication, 1976.

*Not tested.

*Not assigned to a lytic group.

*The indicated frequency denotes that no transformants were recovered under these conditions.
In preliminary experiments, an enhancement of transformation frequencies was observed when exposure of 8325-4 cells to phage 55 occurred in the presence of normal rabbit serum. Therefore, the effect of 10% normal rabbit serum was determined for all serological group B phages tested. Phages 29, 55, 53, 83A, 85, 95, φ11 and 80α all showed an enhancement of transformation frequencies if exposure to the cells occurred in the presence of 10% normal rabbit serum.

Transduction could not have been responsible for these genetic exchanges because, in all cases, treatment of the DNA with DNase prior to exposure to the cells resulted in the inability to recover Nov^R cells. In addition, none of the bacterial strains that were used to propagate the phages were resistant to novobiocin (10 μg/ml).

Survey of representative strains for the ability to demonstrate competence after exposure to phage

Because 8325-4 could be made competent by exposure to a variety of serological group B phages, the incidence of phage-conferred competence among strains of *S. aureus* was investigated. The propagating strains of the *Staphylococcus* reference typing phages were selected for this survey because they were readily available and represent a diversity of staphylococci. To avoid the possibility of genetic incompatibility, a marker was obtained for each strain (Table 8), and the homologous DNA was used in the transformation procedure. Recipient cultures were harvested from TSB during early log growth and incubated with either phage 55, 53, or 80α before being transformed according to the procedure previously described for 8325-4. All of the propagating strains responded to some
degree (Table 8). These experiments were designed to merely survey a large number of strains for the ability to demonstrate competence after exposure to phage; no attempt was made to optimize the conditions for each strain. Because phage 55 demonstrated high competence-conferring activity toward 8325-4, and lacks lytic activity toward the majority of the propagating strains for the typing phages, an attempt was made to confer competence to all the strains with this phage. Strains that did not show activity with phage 55 were tested with other phages until an active phage was found. Because the propagating strains for the lytic group II phages proved to be transducible with phage 55 (data not shown), this phage was propagated on Ps 55thy-135 and used in an attempt to confer competence to these strains. Some competence-conferring activity was detected toward Ps 71thy-136 and Ps 3Cthy-134 (data not shown); however, the frequencies of transformation in these experiments were low, probably due to the lytic activity of phage 55 toward the propagating strains of the lytic group II phages.

Transduction could not have been responsible for these genetic exchanges because treatment of the DNA with DNase prior to its exposure to the cells resulted in the inability to recover Nov^ and Thy^+ cells. In addition, Ps 55 is not resistant to novobiocin (minimum inhibitory concentration = 1.0 μg/ml), and in no instance could phage 53 or 80α be shown to transduce thy^+ into the strains to which they conferred competence.

Several strains exhibited some degree of inherent competence without preexposure to phage (Table 8). With the exception of Ps 187thy-139 and
Table 8. Responses of the propagating strains for the Staphylococcus reference typing phage to become competent for transformation after exposure to phage.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Selected Marker</th>
<th>Phage b</th>
<th>MOI c</th>
<th>Transformation Frequency a With Phage</th>
<th>Without Phage</th>
<th>% Survival d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps 3Athy-133</td>
<td>Ps 3A</td>
<td>thy^+</td>
<td>80α</td>
<td>65</td>
<td>9.5 x 10^-8</td>
<td>&lt;6.6 x 10^-9e</td>
<td>Nt^f</td>
</tr>
<tr>
<td>Ps 3Cthy-134</td>
<td>Ps 3C</td>
<td>thy^+</td>
<td>80α</td>
<td>60</td>
<td>1.1 x 10^-6</td>
<td>&lt;5.3 x 10^-8</td>
<td>Nt</td>
</tr>
<tr>
<td>Ps 55othy-135</td>
<td>Ps 55</td>
<td>thy^+</td>
<td>53</td>
<td>25</td>
<td>2.3 x 10^-7</td>
<td>&lt;8.1 x 10^-8</td>
<td>100</td>
</tr>
<tr>
<td>Ps 71othy-136</td>
<td>Ps 71</td>
<td>thy^+</td>
<td>53</td>
<td>10</td>
<td>1.1 x 10^-7</td>
<td>&lt;1.4 x 10^-9</td>
<td>10</td>
</tr>
<tr>
<td>Ps 42Dthy-137</td>
<td>Ps 42D</td>
<td>thy^+</td>
<td>80α</td>
<td>85</td>
<td>8.9 x 10^-7</td>
<td>&lt;3.8 x 10^-8</td>
<td>Nt</td>
</tr>
<tr>
<td>Ps 42Ethy-138</td>
<td>Ps 42E</td>
<td>thy^+</td>
<td>80α</td>
<td>55</td>
<td>3.8 x 10^-7</td>
<td>&lt;1.7 x 10^-8</td>
<td>Nt</td>
</tr>
<tr>
<td>Ps 187thy-139</td>
<td>Ps 187</td>
<td>thy^+</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<td>2.1 x 10^-6</td>
</tr>
<tr>
<td>Ps 29</td>
<td>Ps 29nov-142</td>
<td>nov</td>
<td>55</td>
<td>40</td>
<td>3.2 x 10^-7</td>
<td>&lt;9.8 x 10^-9</td>
<td>100</td>
</tr>
<tr>
<td>Ps 52</td>
<td>Ps 52nov-142</td>
<td>nov</td>
<td>55</td>
<td>20</td>
<td>9.5 x 10^-9</td>
<td>5.4 x 10^-9</td>
<td>60</td>
</tr>
<tr>
<td>Ps 52A/79</td>
<td>Ps 52A/79nov-142</td>
<td>nov</td>
<td>55</td>
<td>50</td>
<td>1.1 x 10^-6</td>
<td>&lt;1.2 x 10^-8</td>
<td>45</td>
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<tr>
<td>Ps 80</td>
<td>Ps 80nov-142</td>
<td>nov</td>
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<td>1.0 x 10^-8</td>
<td>100</td>
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<td>Ps 81</td>
<td>Ps 81nov-142</td>
<td>nov</td>
<td>55</td>
<td>30</td>
<td>1.7 x 10^-5</td>
<td>&lt;8.1 x 10^-9</td>
<td>20</td>
</tr>
<tr>
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<td>Ps 6nov-142</td>
<td>nov</td>
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<td>40</td>
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<td>100</td>
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<td>Ps 53</td>
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<td>35</td>
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<td>5</td>
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<td>Ps 75nov-142</td>
<td>nov</td>
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<td>3.7 x 10^-6</td>
<td>1.7 x 10^-8</td>
<td>20</td>
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<td>Ps 77nov-142</td>
<td>nov</td>
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<td>30</td>
<td>9.7 x 10^-8</td>
<td>3.4 x 10^-7</td>
<td>10</td>
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<tr>
<td>Ps 83A</td>
<td>Ps83Anov-142</td>
<td>nov</td>
<td>55</td>
<td>35</td>
<td>4.5 x 10^-7</td>
<td>&lt;9.4 x 10^-9</td>
<td>45</td>
</tr>
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<tr>
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<td>Ps 84nov-142</td>
<td>nov</td>
<td>55</td>
<td>35</td>
<td>$6.5 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-7}$</td>
<td>30</td>
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<tr>
<td>Ps 85</td>
<td>Ps 85nov-142</td>
<td>nov</td>
<td>55</td>
<td>30</td>
<td>$4.5 \times 10^{-6}$</td>
<td>$4.1 \times 10^{-7}$</td>
<td>55</td>
</tr>
</tbody>
</table>

- Expressed as the number of transformants/number of viable cells exposed to the phage.
- Phage/propagating strain: 55/Ps 55; 53/Ps 53; 80α/8325-4.
- Multiplicity of infection (MOI) not intended to represent the optimum.
- Percent of total cells recovered after exposure to the phage.
- Less than the indicated frequency denotes that no transformants were recovered under these conditions.
- Not tested.
Ps 77, however, the transformation frequencies obtained after exposure to the phage were significantly higher than the transformation frequencies without preexposure to the phage. Because of the high inherent competence level demonstrated by Ps 187thy-139, it was not possible to quantify the ability of phages to confer competence to this strain. The relatively low frequency of phage-conferred competence observed with Ps 77 was probably because of the loss of transformed cells; a definite loss of viable cells was noted with this strain after exposure to phage 55.

**Assay for congression**

Congression, the expression after genetic exchange of 2 or more genes located on different DNA molecules, has not been observed in the 8325 transformation system (Pattee and Neveln, 1975). Because phage-conferred competence appeared to require a high MOI it was important to determine if this might facilitate the entrance of more than one piece of DNA into the cell. It was also important to establish that known linkages are preserved, indicating that the integrity of the DNA molecule is being maintained and that the genetic manifestation of natural and phage-conferred competence are equivalent.

Three linkage groups have been established in strain 8325 by transformation (Pattee and Neveln, 1975; Pattee, 1976; Pattee et al., 1977; Kuhl et al., 1978). The thy-101 and thrB106 markers show 2% cotransfer and are clearly unlinked to the nov-142 marker. Cells of 8325thy-101 thrB106 ilv-129 tmm-3110 83As pig+ that do not show inherent competence presumably due to the loss part or all of the φ11 prophage (Pattee et al., 1977), were exposed to phage 55/Ps 55thy-135 at an MOI of 7, 13, 50, and
200 and then exposed to 8325\textsuperscript{nov-142} DNA (100 \(\mu\)g/reaction). Although attempts to transduce \(\text{thy}^+\) into 8325 by using phage 55 were unsuccessful, phage 55 propagated on Ps 55\textsuperscript{thy-135} (which is also Nov\textsuperscript{S}) was used to preclude this possibility. Selection was made for \(\text{Thy}^+, \text{Nov}^+, \text{Thy}^+\text{Thr}^+,\) and \(\text{Thy}^+\text{Nov}^+\) transformants. The Nov\textsuperscript{R} transformants were replicated onto CDS agar devoid of thymine and containing 10 \(\mu\)g novobiocin per ml; Thy\textsuperscript{+} transformants were replicated onto the above medium and also onto CDS agar devoid of thymine and threonine. The results of these transformations (Table 9) showed that the 2\% cotransfer of \(\text{thy}^+\) and \(\text{thr}^+\) was retained at an MOI below 50, and the cotransfer of \(\text{thy}^+\) and \(\text{nov}^+\) could not be demonstrated even at an MOI of 200 and an extremely high concentration of DNA. The dearth of \(\text{Thy}^+\text{Thr}^+\) cotransformants among the Thy\textsuperscript{+} transformants at an MOI of 200 is probably because of a loss of potential transformants at such a high MOI. At an MOI of 200 only approximately 13\% of the cells exposed to the phage survived; at an MOI of 7 approximately 71\% of the cells exposed to the phage survived.

**Optimum conditions for phage-conferred competence**

Because phage 55 conferred a relatively high level of competence to 8325-4 without causing significant lethality, this system was chosen to investigate the experimental conditions that would result in optimum levels of phage-conferred competence.

**Multiplicity of infection (MOI)**

Cells grown to early log phase were exposed to phage lysates at varying MOIs and transformed according to the procedure previously described, using 8325\textsuperscript{nov-142} DNA and
Table 9. Cotransformation of markers located in different linkage groups (thy<sup>+</sup> and nov) and within the same linkage group (thy<sup>+</sup> and thr<sup>+</sup>) at different multiplicities of infection.

<table>
<thead>
<tr>
<th>Selected Marker(s)</th>
<th>MOI</th>
<th>Frequency of Transformation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Transformants Scored</th>
<th>% Cotransfer&lt;sup&gt;a&lt;/sup&gt; Thy&lt;sup&gt;+&lt;/sup&gt;Thr&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Thy&lt;sup&gt;+&lt;/sup&gt;Nov&lt;sup&gt;r&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>thy&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7</td>
<td>4.4 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>494</td>
<td>2.4 (12)&lt;sup&gt;c&lt;/sup&gt; &lt;0.20 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>6.6 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>873</td>
<td>2.1 (18) &lt;0.11 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.1 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>1050</td>
<td>1.7 (18) &lt;0.09 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.3 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>100</td>
<td>&lt;1.0 (0) &lt;1.0 (0)</td>
<td></td>
</tr>
<tr>
<td>thy&lt;sup&gt;+&lt;/sup&gt;thr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7</td>
<td>7.1 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>54</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>9.5 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>54</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.0 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>79</td>
<td>1.6</td>
<td></td>
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<tr>
<td></td>
<td>200</td>
<td>1.6 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>12</td>
<td>1.2</td>
<td></td>
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<tr>
<td>nov</td>
<td>7</td>
<td>1.2 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>2001</td>
<td>&lt;0.05 (0)</td>
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<td>13</td>
<td>2.1 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>930</td>
<td>&lt;0.10 (0)</td>
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<td>200</td>
<td>4.8 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>924</td>
<td>&lt;0.10 (0)</td>
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</tr>
<tr>
<td>nov thy&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7</td>
<td>5.3 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>0</td>
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<td></td>
<td>13</td>
<td>5.3 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>0</td>
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<tr>
<td></td>
<td>50</td>
<td>5.3 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>0</td>
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<tr>
<td></td>
<td>200</td>
<td>5.3 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>% Cotransfer = AB/A x 100; where A = Number of selected transformants scored and AB = Number of cotransformants detected. % cotransfer for the Thy<sup>+</sup>Thr<sup>+</sup> selection is expressed as the frequency of transformation of Thy<sup>+</sup>Thr<sup>+</sup>/the frequency of Thy<sup>+</sup>.

<sup>b</sup>Frequency expressed as the number of transformants recovered/number of cells exposed to the phage.

<sup>c</sup>Numbers in parentheses indicate the number of cotransformants detected.
selecting for Nov^ transformants. Approximately 5 to 8 phage per cell were needed to achieve maximum levels of competence (Fig. 5). Multiplicities higher than 8 increased the competence level only slightly, and appeared to have some lethal effect on the cells recovered after exposure to the phage.

Phase of growth The expression of competence for transformation (Lindberg et al., 1972; Rudin et al., 1974) and for transfection (Sjöström et al., 1972; Sjöström et al., 1973) has been reported to be confined to the early log phase of growth in cultures that are lysogenic for φ11. Although the φ11-lysogens used in this study did not demonstrate this distinct competence peak (data not shown), in order to optimize experimental conditions, the effect of the growth phase on the efficiency of phage-conferred competence was determined. Cultures (100 ml each in TSB) of 8325-4 were harvested at intervals during the growth phase; the cells were resuspended in 0.85% NaCl, and the density was adjusted to approximately 3 x 10^9 cfu/ml. The cells were exposed to phage 55 lysates at an MOI of approximately 25, and then transformed with DNA isolated from 8325nov-142. The results (Fig. 6) showed that 8325-4 cells were more efficiently transformed if they were harvested in the early log phase of growth. Although the transformation frequencies decreased sharply after the early log phase, the cells remained somewhat responsive to phage-conferred competence during the late log and early stationary phases.

Rate of growth It was observed that φ11-lysogens that show relatively slow growth rates typically demonstrate higher frequencies of
Fig. 5. The effect of multiplicity of infection on the ability of phage 55 to confer competence to 8325-4.

Competence was measured by transformation with saturating concentrations of 8325nov-142 DNA. Frequency of transfection is expressed as the number of Nov$^r$ transformants recovered/number of viable cells exposed to the phage.
Fig. 6. The effect of the phase of growth on the responsiveness of 8325-4 to become competent after exposure to phage 55.

Competence was measured by transformation with a saturating concentration of 8325_{nov-142} DNA. Frequency of transformation (closed circles) is expressed as the number of Nov\textsuperscript{r} transformants recovered/number of viable cells exposed to the phage. Growth was monitored by absorbance readings taken at a wavelength of 540 nm (open circles).
transformation than those that show relatively faster growth rates (data not shown). Therefore, the effect of slowing the rate of growth on the responsiveness of the cells to phage-conferred competence was examined. The growth rate of 8325-4 was slowed by incubating the cells at 30 C; control cultures were incubated at 35 C. Cells were harvested in early log phase, exposed to phage 55 at an MOI of approximately 20, and then transformed with DNA isolated from 8325nov-142. Cells that were incubated at 30 C exhibited a transformation frequency 31% higher than those incubated at 35C (Table 10). Because the cells were harvested in early log phase it is difficult to accurately calculate growth rate constants for these cells; therefore, the time (min) required to reach an O.D. of 0.10 from an initial O.D. of 0.01 is presented. Despite the lower transformation frequency attained by cells incubated at 35 C, the shorter incubation time required to reach early log phase and the general convenience of using this growth temperature dictated that cells be grown at 35 C. However, many of the experiments yet to be described used 8325thy-101 thrB106 ilv-129 tmn-3110 83A pig which yielded a higher frequency of transformation than 8325-4 using phage-conferred competence, presumably because of its slower rate of growth.

**Exposure time to phage** The 5-min exposure time of the cells to the phage lysates used in the transformation protocol was an arbitrarily selected value; 5 min appeared to be sufficient to render the cells competent but not to allow propagation of the phage on the cells if that particular phage was lytic toward 8325-4. To determine the effect of different exposure times of the cells to the phage, 8325-4 cells were
Table 10. Effect of growth rate on the responsiveness of 8325-4 to phage-conferred competence using phage 55 lysates

<table>
<thead>
<tr>
<th>Incubation Temperature</th>
<th>Growth Time(^a) (min)</th>
<th>Transformation Frequency(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 C</td>
<td>165</td>
<td>$1.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>35 C</td>
<td>125</td>
<td>$5.8 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

\(^{a}\)Time required to reach an O.D. of 0.10 from an initial O.D. of 0.01.

\(^{b}\)Expressed as the number of Nov\(^r\) transformants/number of cells exposed to the phage.
exposed to phage 55 lysates that had been diluted into either TSB or suspension medium in order to achieve an initial MOI of about 20. No serum was added to these phage-cell mixtures because of the possibility of affecting the cellular metabolism during extended incubation. The phage-cell mixtures were incubated at 35 C with gentle shaking; at intervals, samples were removed and transformed with DNA isolated from 8325nov-142. When a significant increase in the cell number was observed in these cells, the cells were diluted to approximately $3 \times 10^9$ cells per ml before they were exposed to the phage. Cells exposed to phage 55 in suspension medium showed a slight increase in competence between 5 and 40 min of exposure time. After 40 min, the transformation frequencies decreased, but the cells showed a relatively high level of competence even after 120 min of exposure (Fig. 7). It was also apparent that these cells did not divide significantly in the suspension medium (0.6 generations). However, the cells that were exposed to the phage in TSB showed a precipitous drop in competence between 5 and 40 min of exposure; the transformation frequencies continued to decrease between 40 and 60 min, after which they appeared to stabilize at a very low level. The cells in TSB did divide significantly during the course of the experiment (2.1 generations).

A separate experiment was conducted to observe the effect of exposing 8325-4 cells to phage in suspension medium for shorter periods of time. Cells were incubated in suspension medium as previously described. Samples were removed at intervals between 0 and 60 min of exposure and
Fig. 7. The effect of exposure time to phage 55 lysates on the responsiveness of 8325-4 to demonstrate competence.

Competence was measured by transformation with a saturating concentration of 8325\textsuperscript{nov-142} DNA. Frequency of transformation of cells exposed to phage 55 in suspension medium (closed circles) and cells exposed to phage 55 in TSB (open circles) is expressed as the number of Nov\textsuperscript{r} transformants recovered/number of viable cells exposed to the phage. The growth response of the cells in suspension medium (closed squares) and in TSB (open squares) is expressed as the number of viable cells per ml. Insert: The acquisition of competence by 8325-4 cells exposed to phage 55 in suspension medium. Samples were taken at intervals between 0 and 60 min of exposure to the phage and assayed for the ability to undergo transformation with 8325\textsuperscript{nov-142} DNA.
assayed for the ability to demonstrate competence for transformation, using 8325nov-142 DNA. Fig. 7 (insert) shows that there was a very rapid acquisition of competence which increased between 0 and 20 min of exposure to the phage. This increase probably reflected the time required for complete adsorption of the phage to the cells. The cells continued to demonstrate high levels of competence up to 40 min of exposure, after which the transformation frequencies decreased slightly. These cells did not divide significantly during the course of the experiment (data not shown).

These results suggested that, like the 8325-4(\phi 12) cells that were rendered competent by incubation in 8325nov-142 growth medium, the 8325-4 cells rendered competent by exposure to phage 55 in suspension medium demonstrated a very rapid acquisition of competence. The level of exposure to the phage stabilized, and then decreased slightly. It should be emphasized that although the frequency of transformation decreased slightly after 40 min of exposure, the level of competence exhibited by the cells after 60 min of incubation was relatively high.

It appears from these data that 8325-4 cells rendered competent by exposure to phage 55 in TSB, which is capable of supporting cell division, lose their ability to be transformed much more quickly than cells rendered competent in a medium which cannot support cell division.

Inhibition of Competence

The results so far presented suggested that the serological group B phages, whether originating from prophage induction or from lytic
propagation, confer competence to cells that normally do not become competent by adsorbing to the cell surface and facilitating the uptake of exogenously supplied DNA upon the exposure to calcium ions. If this proposal is correct, it can be inferred that \( \Phi ll \)-lysogens probably become competent by adsorption of free \( \Phi ll \) particles (or \( \Phi ll \) virion components) that are liberated by prophage induction. Therefore, several approaches were used in an attempt to inhibit the expression of competence in a population of cells that demonstrates natural competence.

**Effect of \( \Phi ll \)-antiserum**

A culture of 8325nov-142 was grown to early log phase in TSB; the cells were harvested by centrifugation, washed once in 0.85% NaCl, and resuspended to the original cell density in filtered medium in which 8325-4 cells had grown to early log phase. This medium was selected in order to mimic the natural cultural conditions without the influence of accumulated \( \Phi ll \) virion components. After 9 min of incubation, either normal rabbit serum, \( \Phi ll \)-antiserum, or an equivalent volume of TSB was added. Samples (1.5 ml each) were removed at intervals, diluted into cold (4°C) Tris-maleate buffer, and transfected with a saturating concentration of 80\% DNA. The results of these experiments were complicated by the low neutralizing capacity of the \( \Phi ll \)-antiserum \((k = 70)\) and by the enhancing effect of antiserum on transfection (as on transformation) frequencies. Preliminary experiments demonstrated that it was necessary to continuously supplement the culture with \( \Phi ll \)-antiserum to achieve neutralization of the phage components that were being released into the
medium (presumably by prophage induction). Thus, when samples were removed, an equivalent volume of serum or TSB was added. Figure 8 shows that the addition of either normal serum or \( \phi 11 \)-antiserum resulted in a rapid enhancement of transfection frequencies. This enhancement was sustained in both cultures for at least 40 min. However, upon extended incubation, a decrease in the ability to undergo transfection was evident in the culture that was treated with \( \phi 11 \)-antiserum; the culture treated with normal serum continued to demonstrate high levels of competence. The culture that did not receive any serum showed a steady increase in the ability to undergo transfection. This increase was probably due to an accumulation of \( \phi 11 \) virion components in the medium. Although there was a significant decrease in transfection frequencies after 40 min of exposure to the \( \phi 11 \)-antiserum, it was not possible to completely inhibit the competence of 8325\text{nov-142} under these conditions.

In an attempt to circumvent the problem of antiserum enhancement of transfection frequencies, the gamma-globulin-containing fraction of the serum was precipitated with ammonium sulfate and used in an attempt to neutralize the \( \phi 11 \)-components without enhancing the frequency of transfection. However, as described in subsequent experiments (see p. 146), Enhancement of Transfection Frequencies with Normal Rabbit Serum) the competence-enhancing factor of the serum coprecipitated with the gamma-globulin-containing fraction.
Fig. 8. The effect of incubation with φ11-antiserum on the expression of competence by 8325nov-142.

Cells were inoculated into filtered 8325-4 growth medium, and at 9 min of incubation, each culture received either φ11-antiserum (closed circles), normal rabbit serum (open circles), or TSB (triangles). Competence was measured by transfection with a saturating concentration of 80α DNA. Frequency of transfection is expressed as the number of 80α plaques observed/number of viable cells exposed to the DNA.
Effect of sodium dodecyl sulfate (SDS)

Because attempts to inhibit the expression of competence in cultures of 8325nov-142 by treatment with φ11-antiserum met with limited success, the problem was approached from a different perspective. If the 8325nov-142 cells were adsorbing φ11 components from the medium and thus being rendered competent, it should have been possible to remove the φ11 components from the cell surfaces and thus render the cells non-competent. Assuming that the attractions between the cell surface and the phage components were electrostatic in nature, treatment of the cells with an ionic detergent, such as sodium dodecyl sulfate (SDS), should result in dissociation of the φ11 components from the cell surfaces. A TSB culture (100 ml) of 8325nov-142 that had grown to early log phase was divided into 10-ml samples, and the cells were removed by centrifugation (3,400 x g, 7 min, room temperature). The cells were resuspended in TSB containing varying concentrations of SDS. These cultures were incubated at 35 C with shaking for 15 min; after incubation, the cells were removed by centrifugation as before and washed twice with Tris-maleate buffer. The cells were transfected with φ80α DNA and assayed for both transfectants and cell viability. The results (Fig. 9) showed that although high concentrations of SDS were cytotoxic (presumably due to disruption of the cytoplasmic membrane), concentrations below 0.006% showed no effect on cell viability. However, cells treated with 0.004% SDS exhibited a reduction in transfection frequency of 44%. Treatment with 0.008% SDS resulted in a 26% reduction in viable cells, but this corresponded to a 67% reduction in transfection frequency.
Fig. 9. The effect of SDS on the expression of competence by 8325nov-142.

Cells were incubated with varying concentrations of SDS in TSB at 35 C for 15 min. Competence was measured by transfection with 80α DNA. Frequency of transfection (open circles) is expressed as the number of 80α plaues observed/number of viable cells recovered from the treatment (open circles).
It was subsequently shown that treatment of the 8325nov-142 growth medium with 0.004% SDS resulted in a reduction of 75% in the competence-conferring activity of this medium (data not shown). However, it could not be determined from these experiments if the reduction was attributable to failure of the φ11 components to adsorb to the cell surface or to dissociation of the protein subunits of the virus components (or to both).

Effect of Pronase

Although the treatment of competent cells with SDS appeared to be effective in rendering them noncompetent, the probability of imparting nonspecific lethal effects to the cell, in general, and to the cytoplasmic membrane, in particular, limited its usefulness as a tool to study the phenomenon of natural competence. Because the competence-conferring component in the 8325nov-142 medium did not appear to be an intact φ11 particle (Table 5), but was neutralizable with φ11-antiserum (Table 6), it was suspected that this component was probably an attachment organelle of the phage. Therefore, digestion of the component away from the cell surface was attempted. A TSB culture (100 ml) of 8325nov-142 was grown to early log phase and divided into 10-ml samples. To each sample was added varying concentrations of Pronase. Cells were treated with Pronase at 35 C with gentle shaking for 20 min. The cells were removed by centrifugation (3,400 g, 7 min, room temperature), transfected with 80 α DNA and assayed for transfectants and cell viability. The results (Fig. 10) showed that the frequency of transfection decreased continuously as the concentration of Pronase was increased; Pronase.
Fig. 10. The effect of Pronase on the expression of competence by 8325nov-142.

Cells were treated with varying concentrations of Pronase at 35°C for 20 min. Competence was measured by transfection with a saturating concentration of 80α DNA. Frequency of transfection (open circles) is expressed as the number of 80α plaques observed/number of viable cells recovered from the treatment (closed circles).
concentrations greater than 2000 μg/ml resulted in a complete inhibition of competence as measured by transfection. Cell viability was not affected by the Pronase.

In addition to rendering the 8325nov-142 cells noncompetent with Pronase, the 8325nov-142 growth medium was also pretreated with Pronase before being used to confer competence to 8325-4(φ12). To 10-ml samples of the growth medium were added varying concentrations of Pronase. The medium was digested at 35 C for 20 min and then inoculated with 8325-4(φ12) cells that had been harvested in early log phase to attain a cell density comparable to that observed during early log phase (O.D. approximately 0.20). The 8325-4(φ12) cells were removed by centrifugation (3,400 x g, 7 min, room temperature), transfected with 80α DNA and assayed for transfectants and cell viability. The results (Fig. 11) showed that pretreatment of the 8325nov-142 medium with Pronase resulted in the inactivation of the competence-conferring activity of this medium. Pronase concentrations higher than 1000 μg/ml inactivated all detectable levels of competence-conferring activity. Cell viability was not affected by the Pronase.

It was not possible to determine from these experiments whether the cells were losing the ability to undergo transfection due to digestion of the φ11 component or to digestion of some other proteinaceous DNA-binding site. Therefore, the effect of treating the 8325-4(φ12) cells with 1000 μg of Pronase per ml before and after exposure to the 8325nov-142 medium was examined. This experiment was essentially the same as those
Fig. 11. The effect of treating the 8325nov-142 growth medium with varying concentrations of Pronase on the ability of the medium to confer competence to 8325-4(φ12).

Competence was measured by transfection with a saturating concentration of 80α DNA. Frequency of transfection (open circles) is expressed as the number of 80α plaques observed/number of viable cells recovered from the treatment (closed circles).
described previously in this section; the individual sample treatments are described in Table 11. The results (Table 11) showed that 8325-4 (φ12) cells treated with Pronase after exposure to the 8325nov-142 growth medium showed a reduction in the transfection frequency of 93%. However, if the 8325-4(φ12) cells were treated with Pronase before exposure to the 8325nov-142 growth medium, no reduction in the transfection frequency was observed.

Because of the similarities between the growth media from 8325nov-142 and 8325-4(φ11) in conferring competence to 8325-4(φ12) (Tables 4 and 6), these experiments were repeated using the growth medium from 8325-4(φ11). The individual sample treatments are described in Table 12. The results (Table 12) showed that when the 8325-4(φ11) growth medium was treated with 1000 µg of Pronase per ml before being used to confer competence to 8325-4(φ12), the competence-conferring activity of the medium was reduced by 99%. The 8325-4(φ12) cells treated with Pronase after exposure to the 8325-4(φ11) growth medium exhibited an 82% reduction in transfection frequency; however, 8325-4(φ12) cells treated with Pronase before exposure to the 8325-4(φ11) growth medium showed no reduction in transfection frequency.

In a separate experiment, the 8325-4(φ11) growth medium was pretreated with 1000 µg of Pronase per ml at 35 C for 30 min. To this digested medium was added 8325-4(φ12) cells that had been grown to early log phase. The cells were incubated in this medium at 35 C with shaking for 20 min, removed by centrifugation, and transfected with 80α DNA.
Table 11. Effect of treating 8325-4(φ12) cells with 1000 μg Pronase per ml before and after exposure to 8325nov-142 growth medium. Cells were assayed for competence by transfection with 80α DNA.

<table>
<thead>
<tr>
<th>Treatment before Transfection</th>
<th>Frequency of Transfection$^a$</th>
<th>Percent Reduction$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8325-4(φ12) incubated in 8325nov-142 growth medium for 20 min (Control)</td>
<td>$2.1 \times 10^{-6}$</td>
<td>0</td>
</tr>
<tr>
<td>8325-4(φ12) incubated in 8325nov-142 growth medium that had been treated with Pronase for 20 min</td>
<td>$1.8 \times 10^{-8}$</td>
<td>99.1</td>
</tr>
<tr>
<td>8325-4(φ12) treated with Pronase 20 min, washed with 0.85% NaCl</td>
<td>$&lt;2.4 \times 10^{-9}$$^d$</td>
<td>-9.5$^e$</td>
</tr>
<tr>
<td>8325-4(φ12) treated with Pronase 20 min, washed with 0.85% NaCl, then incubated in 8325nov-142 growth medium for 20 min.</td>
<td>$2.3 \times 10^{-6}$</td>
<td>-9.5$^e$</td>
</tr>
<tr>
<td>8325-4(φ12) incubated in 8325nov-142 growth medium 20 min, treated with Pronase for 20 min, then washed with 0.85% NaCl</td>
<td>$1.4 \times 10^{-7}$</td>
<td>93.3</td>
</tr>
</tbody>
</table>

$^a$Expressed as the number of transfectants/number of viable cells exposed to the DNA.

$^b$Control frequency - Test frequency $\times 100$

$^c$All incubations carried out at 35 C with gentle shaking.

$^d$Less than the designated frequency denotes no transfectants were observed.

$^e$Negative value indicates that the test frequency was larger than the control frequency.
Table 12. Effect of treating 8325-4(φ12) cells with 1000 μg Pronase per ml before and after exposure to 8325-4(φ11) growth medium. Cells were assayed for competence by transfection with 80α DNA.

<table>
<thead>
<tr>
<th>Treatment before Transfection</th>
<th>Frequency of Transfection&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent Reduction&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8325-4(φ12) incubated&lt;sup&gt;c&lt;/sup&gt; in 8325-4(φ11) growth medium for 20 min (Control)</td>
<td>5.6 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>8325-4(φ12) incubated in 8325-4(φ11) growth medium that had been treated with Pronase for 20 min</td>
<td>7.2 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>99.2</td>
</tr>
<tr>
<td>8325-4(φ12) treated with Pronase 20 min, washed with 0.85% NaCl, then incubated in 8325-4(φ11) growth medium 20 min</td>
<td>6.2 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>-10.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>8325-4(φ12) incubated in 8325-4(φ11) growth medium 20 min, treated with Pronase for 20 min, then washed with 0.85% NaCl</td>
<td>1.0 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>82</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as the number of transfectants/number of viable cells exposed to the DNA.

<sup>b</sup>\[
\frac{\text{Control frequency} - \text{Test frequency}}{\text{Control frequency}} \times 100
\]

<sup>c</sup>All incubations carried out at 35 C with gentle shaking.

<sup>d</sup>Negative value indicates that the test frequency was larger than the control frequency.
The digested medium was also assayed for infectious phage particles by using 8325-4 as an indicator. The Pronase-digested medium showed a 96% reduction in competence-conferring activity, but only a 26% reduction in the number of infectious phage particles.

The inactivation by Pronase of the competence-conferring activity of the growth medium from 8325nov-142 and 8325-4(φ11), and the insensitivity to Pronase of the infectious phage particles in the latter medium, suggested that the competence-conferring ability of the 8325-4(φ11) medium is attributable entirely to a φ11-component and that the infectious φ11 particles present do not contribute to the activity. Furthermore, the inhibition of the expression of competence in 8325nov-142 by treatment with Pronase supported the inference that competence in φ11-lysogens is due to the adsorption of φ11-components from the medium.

Detection of Competence-Conferring Components in Phage Lysates

The similarities between competence demonstrated by φ11-lysogens and competence conferred by externally supplied phage suggested that these two modes of the expression of competence are probably manifestations of the same physiological mechanism. This mechanism appeared to involve the adsorption of phage particles (or virion components) which alter the cell surface in an undetermined manner and facilitate the uptake of DNA. However, to this point the early phage gene theory of Sjöström and Philipson (1974) had not been completely refuted. Therefore, it was necessary to conclusively demonstrate that the phage genome does not play a direct role in the expression of competence. In addition, if
natural competence demonstrated by \( \Phi l l \)-lysogens and phage conferred competence are identical, it should be possible to isolate the competence-conferring component from the phage lysates and to determine its identity.

**Ultraviolet (UV) irradiation of phage lysates**

At the routine test dilution phage 55 is not lytic toward 8325-4. This insensitivity is probably due to a restriction-modification system (Stobberingh and Winkler, 1976), although lysogenic immunity may also play a role. If the competence-conferring ability of phage 55 is attributable to an early gene as described by Sjöström and Philipson (1974) for \( \Phi l l \), it is possible that a rapid expression of the gene might occur before the infecting phage genome is either restricted or repressed, resulting in the expression of competence. To investigate the role that the phage 55 genome plays in the expression of competence, a lysate of phage 55 was diluted into suspension medium to attain a titer of approximately \( 1 \times 10^{10} \) pfu/ml; this titer resulted in an MOI of about 5 when used to confer competence to 8325-4. Approximately 25 ml were placed in a heavy glass petri-dish (8.5 cm diameter), horizontally agitated, and irradiated with a 15 W germicidal lamp (Champion G15T8) at a dose-rate of \( 1 \text{ J/m}^2/\text{sec} \). Samples were removed at cumulative intervals and assayed under reduced illumination for both phage survival and competence-conferring activity. Fig. 12 shows that although 99.999% of the phage were inactivated after 5 min of irradiation the competence-conferring activity of the phage did not change significantly.
Fig. 12. The effect of UV-inactivation on the ability of a phage lysate to confer competence to 8325-4.

Phage were irradiated at cumulative intervals at a dose-rate of 1 J/m²/sec. Samples were assayed for surviving phage (open circles) and competence-conferring activity measured by transformation with a saturating concentration of 8325nov-142 DNA. Frequency of transformation (closed circles) is expressed as the number of Nov⁺ transformants recovered/number of viable cells exposed to the phage.
In a separate experiment, a phage 55 lysate was irradiated as described above until 99.5% of the phage had been inactivated. These UV-inactivated phage were used to determine the optimum MOI needed to achieve maximum competence. Fig. 13 shows that when the MOI is expressed in terms of the infectious phage particles in the unirradiated lysate, the relationship between MOI and competence-conferring activity was similar to that observed with the unirradiated lysate (Fig. 5). Approximately 10 phage per cell were needed to achieve a saturating level of competence. MOIs higher than ten increased the level of competence only slightly. If, however, the MOI is expressed in terms of the infectious phage particles remaining after irradiation, approximately 0.017 phage per cell were needed to achieve the same level of competence.

**Competence-conferring ability of purified phage**

The ability of phage 55 lysates containing UV-inactivated phage particles to confer competence to 8325-4 demonstrated that the phage genome does not play a direct role in phage conferred competence. In addition, during the course of this study it was observed that lysates containing equal numbers of pfu can differ significantly in their competence-conferring activity. Because the competence-conferring activity of the 8325-4(φ11) growth medium appeared to be due entirely to a φ11 virion component, it was necessary to determine if purified infectious phage could confer competence.

Phage 80α was selected for this study because of its extreme stability in CsCl. The phage was propagated on a large scale on 8325-4
Fig. 13. The effect of UV-inactivation of phage 55 on the optimum MOI needed to confer competence to 8325-4.

The phage were irradiated at a dose-rate of 1 J/m²/sec until 99.5% of the phage had been inactivated and then used to confer competence to 8325-4. Competence was measured by transformation with a saturating concentration of 8325nov-142 DNA. Frequency of transformation is expressed as the number of Nov^T transformants recovered/number of viable cells exposed to the phage.
and the majority of the pfu were removed by centrifugation (48,700 x g, 2 h, 20 C). The pelleted phage were purified by banding in an isopycnic CsCl gradient (45% w/w, 61,000 x g, 48 h, 20 C). The CsCl was removed by dialysis and the purified phage as well as the crude lysate were diluted into TSB to attain a titer of approximately 10^{10} pfu/ml. The diluted crude lysate, the diluted purified phage, and the supernatant from which the phage had been pelleted were used to confer competence to 8325thy-101 thrB106 ilv-129 tmm-3110 83AB pig^{+}; this organism was selected because it showed high levels of competence when exposed to phage 55. After exposure to the phage, the cells were transformed with 8325nov-142 DNA. The results (Table 13) demonstrated that the crude lysate showed relatively high levels of competence-conferring activity, but the supernatant showed greater activity although it contained fewer pfu. In addition, the phage that had been purified by banding in CsCl showed very low competence-conferring activity. Similar results were obtained with phage 55 that had been purified by banding in CsCl (data not shown); however, the instability of phage 55 in CsCl proved to be detrimental to obtaining reliable data using this phage.

Effect of Pronase on the competence-conferring activity of the 80α supernatant

In order to determine if the competence-conferring activity of the 80α supernatant was more susceptible to inactivation by Pronase than the remaining pfu, 1.0-ml samples of the lysate were treated with varying concentrations of Pronase for 30 min at 35 C. These samples were then
Table 13. Ability of 80α to confer competence to 8325thy-101 thrB106 ilv-129 tnm-3110 83AS pig+. Comparison of the activities of the crude lysate, lysate supernatant, purified phage.

<table>
<thead>
<tr>
<th>Phage Preparation</th>
<th>PFU/ml</th>
<th>Transformation Frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MOI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percent Activity of Crude Lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate, diluted 1:20 into TSB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>$1.1 \times 10^{10}$</td>
<td>$8.5 \times 10^{-7}$</td>
<td>15</td>
<td>--</td>
</tr>
<tr>
<td>Supernatant from centrifuged&lt;sup&gt;d&lt;/sup&gt; crude lysate</td>
<td>$1.1 \times 10^{9}$</td>
<td>$4.3 \times 10^{-6}$</td>
<td>1.5</td>
<td>500</td>
</tr>
<tr>
<td>Purified by banding in CsCl, then diluted 1:800 into TSB</td>
<td>$1.0 \times 10^{10}$</td>
<td>$1.3 \times 10^{-8}$</td>
<td>14</td>
<td>1.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as transformants recovered/number of viable cells exposed to phage.

<sup>b</sup> Multiplicity of infection.

<sup>c</sup> Trypticase soy broth.

<sup>d</sup> 48,700 x g, 2 h, 20 C.
assayed for pfu and used to confer competence to 8325thy-101 thrB106 ilv-129 tmn-3110 83A8 pig+. After exposure to the phage, the cells were transformed with 8325nov-142 DNA. The results (Fig. 14) showed that as the Pronase concentration was increased, the numbers of infectious phage particles decreased slightly, but the competence-conferring activity decreased sharply. Treatment of the supernatant with 3000 μg of Pronase per ml resulted in inactivation of 56% of the pfu and 93% of the competence-conferring activity.

Attempts to dissociate phage particles

The competence-conferring activity of the 80α lysate and the growth media from φ11-lysogens appeared to be due entirely to a phage component with no contribution from infectious phage particles. Therefore, attempts were made to dissociate phage particles and, thus, generate more competence-conferring activity in the phage lysate.

Effect of sonication

A lysate of phage 55 that contained a high titer of infectious phage particles and also showed high competence-conferring activity was dialyzed against two 12-h changes of suspension medium (500 ml each) to remove some of the viscosity. The dialyzed lysate (3.5 ml) was then sonicated in an ice bath at cumulative intervals (15 sec each) at 50% output of a Biosonic II sonicator equipped with a standard probe. After a total of 5.0 min of sonication, the lysate was assayed for remaining infectious particles and for competence-conferring activity toward 8325-4. After exposure to the phage, the cells were transformed with 8325nov-142 DNA. Although the sonication reduced the
Fig. 14. The effect of treatment with varying concentrations of Pronase on the ability of the 80α lysate supernatant to confer competence to 8325thy-101 thrB106 ilv-129 tmn-3110 83A® pig⁺.

Competence was measured by transformation with a saturating concentration of 8325nov-142 DNA. Frequency of transformation (closed circles) is expressed as the number of Nov⁺ transformants recovered/number of viable cells exposed to the phage. The effect of Pronase on the survival of infectious phage particles (pfu) is also indicated (open circles).
FREQUENCY OF TRANSFORMATION $\times 10^{-5}$

PRONASE CONCENTRATION (µg/ml)

PFU PER ML $\times 10^7$
number of infectious phage by 99.6% (0.4% survival), no change in the competence-conferring activity was observed (Table 14).

**Effect of repeated freezing and thawing** During the course of this investigation it was observed that lysates of φ11 in TSB appear to be rather unstable, undergoing a reduction in titer of approximately 50% (½ log) during the first 24 h of storage at 4 C. The apparent fragility of this phage suggested that it would be more susceptible to disruption by physical methods than other phages. A φ11 lysate was subjected to a series of 15 repeated cycles of freezing (dry ice-acetone) and thawing (water bath at 35 C). After the 15th thaw, the lysate was assayed for infectious phage particles; the 15 cycles of freezing and thawing resulted in only a 48% reduction in infectious phage particles (Table 14). Because of the apparent inefficiency of this method, the phage lysate was not tested for the ability to confer competence.

**Effect of dimethyl sulfoxide** It had been reported that 67% dimethyl sulfoxide (DMSO) disrupts T-even bacteriophage particles into component parts (Cummings et al., 1968). Therefore DMSO (Crown Zellerman Corp., Camas, WA) was added slowly (4 drops/min) to a lysate of 80α (1.3 X 10¹¹ pfu / ml). The temperature of the lysate was maintained at approximately 10 C by occasionally placing it in an ice bath. When 67% DMSO was reached, the lysate was dialyzed against two 12-h changes of suspension medium (1000 ml each) at 4 C. The lysate was assayed for
Table 14. Attempts to dissociate phage lysates in order to generate competence-conferring components\(^a\) from infectious phage particles

<table>
<thead>
<tr>
<th>Phage Lysate</th>
<th>Treatment</th>
<th>pfu/ml</th>
<th>Transformation Frequency(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage 55</td>
<td>None</td>
<td>4.5 x 10(^{10})</td>
<td>1.5 x 10(^{-6})</td>
</tr>
<tr>
<td>Phage 55</td>
<td>Sonicated 5.0 min</td>
<td>1.8 x 10(^{8})</td>
<td>1.3 x 10(^{-6})</td>
</tr>
<tr>
<td>$\phi$11</td>
<td>None</td>
<td>2.7 x 10(^{11})</td>
<td>NT(^c)</td>
</tr>
<tr>
<td>$\phi$11</td>
<td>Repeated freezing and thawing</td>
<td>1.4 x 10(^{11})</td>
<td>NT</td>
</tr>
<tr>
<td>80$\alpha$</td>
<td>Diluted 67% with TSB</td>
<td>4.3 x 10(^{10})</td>
<td>2.2 x 10(^{-6})</td>
</tr>
<tr>
<td>80$\alpha$</td>
<td>67% DMSO</td>
<td>3.0 x 10(^{8})</td>
<td>4.2 x 10(^{-7})</td>
</tr>
</tbody>
</table>

\(^a\) Phage preparations used to confer competence for transformation to 8325-4, using 8325nov-142 DNA.

\(^b\) Number of transformants recovered/number of cells exposed to the phage.

\(^c\) Not tested.
infectious phage particles and for competence-conferring activity toward 8325-4. After exposure to the phage, the cells were transformed with 8325nov-142 DNA. Although the DMSO treatment reduced the number of infectious phage particles by approximately 99%, it resulted in a reduction of competence-conferring activity of 81% (Table 14). Similar results were obtained with a φ11 lysate that was treated with 67% DMSO (data not shown).

Attempts to identify a mutant with increased competence-conferring activity

The failure to generate competence-conferring components by dissociating intact phage particles suggested that the phage units created during phage assembly might not be identical to those created during physical dissociation of virions. Therefore, attempts were made to identify a mutant phage that demonstrated increased competence-conferring activity, presumably due to a defective virion component that is not involved with conferring competence or to a defective assembly process.

Suppressor-sensitive mutants The suppressor-sensitive mutants of φ11 isolated by Kretschmer and Egan (1973, 1975) were obtained from Sidney Cohen, Michael Reese Medical Center, Chicago, IL, along with the suppressor host mutant of strain 8325. These phage mutants were provided in the form of lysogens of the suppressor host mutant. Lyso-gens carrying phage mutants that were believed to be defective in head-formation (susH47, susM28; Kretschmer and Egan, 1975) were grown to early log phase in TSB (100 ml) at 35 C. The cells were removed by
entrifugation, and the media were assayed for pfu by using both the suppressor host mutant and 8325-4 as indicators. The medium from both lysogens yielded phage particles that plated with nearly equal efficiency on both the suppressor host mutant and on 8325-4. Thus, it was concluded that this suppressor-sensitive system was too leaky to be utilized in preparing copious amounts of competence-conferring component. This leakiness can probably be explained by the subsequent demonstration of the suppressor host mutant as missense suppressor instead of a nonsense suppressor as originally proposed (J. Barry Egan, University of Adelaide, Adelaide, South Australia, personal communication).

Temperature-sensitive mutants In addition to the suppressor-sensitive mutants of φ11 isolated and characterized by Kretschmer and Egan, 18 temperature-sensitive mutants of φ11 (ts φ11) were obtained from Richard Novick, The Public Health Research Institute of the City of New York, Inc., New York, NY. A lysate of each mutant was prepared by serial propagation on 8325-4 at the permissive temperature (30 C).

Attempts were made to obtain lysogens of the ts φ11 mutants by incubating log phase TSB cultures of 8325-4 with each ts φ11 mutant at 30 C until lysis occurred and resistant cells proliferated. Resistant cells were harvested by centrifugation, washed and resuspended in 0.85% NaCl, and streaked repeatedly onto TSA until no further lytic activity was detected. In addition, resistant cells from the center of turbid plaques formed on 8325-4 at 30 C were streaked repeatedly onto TSA until no further lytic activity could be detected. Each suspended lysogen was then radially streaked onto BHI agar, and after incubation at 30 C, each
plate was replicated onto TSA that had been surface inoculated with 8325-4. Lysogens were denoted by a zone of lysis surrounding the streaked cells and were retained for further investigation.

After repeated attempts, stable lysogens were isolated for only 5 of the 18 ts φ11 mutants. These lysogens were screened for defective phage production by assaying for lysogens with a high frequency of transfection, but a low number of infectious phage particles in the medium when incubated at the nonpermissive temperature (42°C). None of the 5 lysogens showed this expected correlation. By contrast, the 8325-4(φ11) cultures used as a control showed approximately the same frequency of transfection and phage production at 42°C and at 30°C.

Attempts were also made to obtain lysates reduced in pfu and increased in competence-conferring activity by propagating the ts φ11 on 8325-4 at the nonpermissive temperature. TSB cultures of 8325-4 that had reached the log phase of growth were infected with each ts φ11 mutant at an MOI of about 5. The cells were incubated at 42°C with gentle shaking, and the turbidity was monitored by O.D. readings against an uninfected control culture. When (and if) the optical density stabilized or decreased, the cells were removed by centrifugation, and the supernatant was passed through a sterile Selas filter. This approach did not prove to be satisfactory because most of the ts mutants failed to retard growth and the probability of selecting for wild-type revertants upon prolonged incubation was a major concern.

A modification of the above procedure was then employed. After infection, the cells were incubated with shaking at 42°C for 40 min.
This is the length of time required to see stabilization with a subsequent decrease in O.D. when 8325-4 is infected with wild-type P11, and infection is allowed to proceed at 42 C. After 40 min of incubation, 2.0 μg of lysostaphin per ml was added, and incubation was continued until the cultures lysed (usually about 10 min). The unlysed cells were removed by centrifugation, and the lysates were passed through sterile Selas filters. The lysates were assayed for pfu at 30 C using 8325-4 as an indicator and for competence-conferring activity toward 8325-4, using 8325nov-142 DNA. Although the number of pfu differed greatly from one lysate to another, all of the lysates were relatively ineffective at conferring competence to 8325-4 (data not shown).

Separation of pfu from competence-conferring components in 80α lysates

The inability to obtain a lysate enriched in competence-conferring component by physical or genetic manipulation of phage initiated an attempt to isolate the competence-conferring component from a wild-type lysate. Phage 80α was selected for this purpose because of its extreme stability and because it confers high levels of competence to 8325-4 despite its lytic nature toward this strain. This latter property suggests that in conventional lysates of 80α, the concentration of competence-conferring component is rather high. The following procedures were performed on phage 80α lysates that were centrifuged (48,700 x g, 2 h, 20 C) to remove a majority (99.9%) of the pfu; this preparation will be referred to as a centrifuged lysate for the remainder of this dissertation. In addition, in the following procedures, the
phage preparations were tested for competence-conferring activity using 8325thy-101 thrB106 ilv-129 tmn-3110 pig^{+} 83A^{S} (ISP 173) as the recipient and 8325nov-142 as the source of donor DNA.

**Ammonium sulfate precipitation** To 300 ml of centrifuged lysate was added crystalline (NH_{4})_{2}SO_{4} in 5% (w/v) increments ranging from 10-40%. After each addition, the lysate was stirred at room temperature for 30 min, and then the precipitate was removed by centrifugation (10,000 x g, 30 min, 4 C). A 10-ml sample of the supernatant was then removed and dialyzed against suspension medium (4 12-h changes, 500 ml each) at 4 C. The dialyzed samples were corrected for volume changes using suspension medium and assayed for pfu and competence-conferring activity. The results (Fig. 15) showed that although the competence-conferring component could be concentrated with (NH_{4})_{2}SO_{4} precipitation, it coprecipitated with the pfu.

**Precipitation with polyethylene glycol** Polyethylene glycol (PEG) has been used to concentrate bacteriophage from large volumes of lysate (Yamamoto et al., 1970). This procedure was used in this investigation to examine the possibility that the infectious phage particles might be removed from the lysate leaving the competence-conferring components in solution. The centrifuged lysate (500 ml) was made 0.5 M with respect to NaCl, and then 10% (w/v) of PEG 6000 (Union Carbide, New York, NY) and dissolved at room temperature. The mixture was then placed at 4 C for 5 h, centrifuged (10,000 x g, 20 min, 4 C) to remove the PEG-precipitated material which was resuspended in 20 ml of suspension.
Fig. 15. Coprecipitation of competence-conferring activity and pfu from a centrifuged 80x lysate by (NH₄)₂SO₄.

Supernatants were assayed for pfu (open circles) and used to confer competence to 8325thy-101 thrB106 ilv-129 tnrn-3110 83A pig⁺. Competence was measured by transformation with a saturating concentration of 8325nov-142 DNA. Frequency of transformation (closed circles) is expressed as the number of Nov⁺ transformants recovered/number of viable cells exposed to the samples.
medium. Both the supernatant and the resuspended pellet were passed through Selas filters and assayed for both pfu and competence-conferring activity. Again, the competence-conferring components coprecipitated with the pfu (data not shown).

**Ultrafiltration**

The φ11 virion has morphological features that classify it in Bradley's group B (Bradley, 1967); these include a hexagonal head measuring about 50 nm in diameter and a flexible tail measuring about 156 nm long and 8.1 nm wide (Brown et al., 1972). Therefore, assuming that the competence-conferring component is a subvirion particle, it should have been possible to separate the competence-conferring component from infectious phage particles by ultrafiltration. Filters (30 nm pore size) were obtained from Nucleopore Corp., Pleasanton, CA (Nucleopore N 003 CPR). These filter were adapted to fit an Amicon model 52 ultrafiltration cell (Amicon Corp., Lexington, MA) by cutting the filter and adhering it to an Amicon filter backing from which the Amicon filter had been removed. Centrifuged lysate (24 ml) that had been concentrated to one-fifth of the original volume with Lyphogel (Gelman Instruments, Ann Arbor, MI) was passed through the filter using 60 lb/in$^2$ of N$_2$ applied for 4 h at 4 C. The filtrate was then passed through a Selas filter and assayed for pfu and competence-conferring activity. While the filtration reduced the pfu by 99.4%, it reduced the competence-conferring activity by only 42% (data not shown). The reduction in competence-conferring activity was presumably due to obstruction of some of the competence-conferring components at the filter surface. The
filtrate, however, contained a significant number of residual pfu; the reason for this was not apparent.

Isopycnic CsCl gradients Intact serological group B Staphylococcus aureus phages have a density of approximately 1.50 g/cm$^3$ (Rosenblum and Tyrone, 1964). Because the competence-conferring component appeared to have properties of a proteinaceous subvirion component, it should have a density of approximately 1.30 g/cm$^3$ (Beard et al., 1972). Therefore, an isopycnic CsCl gradient was used to separate the competence-conferring component from the infectious phage particles. The centrifuged lysate was concentrated to one-fifth the original volume with Lyphogel, and 32% (w/w) technical grade CsCl (Apache Chemicals, Seward, IL) was added and allowed to completely dissolve. The actual density was adjusted to 1.30 g/cm$^3$ using refractometry. The phage-CsCl mixture was placed in 4-ml polyallomer centrifuge tubes, and micule density markers (Beckman Instruments, Inc., Palo Alto, CA) were added to expedite the identification of fractions (Griffith et al., 1967). The tubes were centrifuged (88,500 x g, 48 to 72 h, 20 C), and the resulting gradients were fractionated into 0.5-ml aliquots from the top of the gradient. Corresponding fractions from all gradients were pooled and dialyzed against suspension medium (four 12-h changes, 500 ml each) at 4 C. The fractions were assayed for both pfu and competence-conferring activity. The distribution of pfu and competence-conferring activity isolated in each fraction of the CsCl gradient are shown in Fig. 16. The majority of the competence-conferring activity was found 2.0 to 2.5 ml from the top of the gradient; the 2.0-ml
Fig. 16. Isolation of a fraction enriched for competence-conferring activity from a centrifuged 80a lysate by buoyant density centrifugation in an isopycnic CsCl gradient.

Fractions were assayed for pfu (triangles) and the ability to confer competence to 8325thy-101 thrB106 ilv-129 tmn-3110 83A^ pig'. Competence was measured by transformation with a saturating concentration of 8325nov-142 DNA. Frequency of transformation (circles) is expressed as the number of Nov^ transformants recovered/number of viable cells exposed to the fraction.
fraction also contained the 1.300 g/cm$^3$ density marker. No competence-confering activity was detected in the 0.5 to 1.0-ml fractions. Because of the limited length of the gradient, most of the pfu were pelleted as was the 1.500 g/cm$^3$ marker. However, all of the upper fractions contained residual infectious particles, although the numbers increased in the lower fractions. This was probably because of aggregation of intact virions with other material in the lysate, resulting in a change of density.

To confirm the phage-origin of the competence-confering activity in the upper fractions of the CsCl gradient, the material from the 2.0-ml fraction was incubated with 10% serological group B antiserum ($\phi 11$-antiserum, $k = 70$) at 35 C for 30 min. The serum-neutralized fraction was then assayed for competence-confering activity, using 8325$\text{thy-101 thr-B106 ilv-129 tmn-3110 83A}^S \text{ pig}^+$ and a saturating concentration of 8325$\text{nov-142 DNA}$. The results (not shown) demonstrated complete inhibition of the competence-confering activity of this fraction by preincubation with immune serum.

**Ficoll gradients**  The competence-confering component was further purified by rate-zonal centrifugation through a Ficoll gradient. Ficoll 400 (Pharmacia, Uppsala, Sweden) was dissolved in TSB to attain concentrations of 5–25% (w/v) in 5% increments. Gradients were prepared by carefully layering the cold (4 C) Ficoll solutions (2 ml each) in order of decreasing concentration on top of a 0.5-ml cushion of 50% Ficoll in a 12.5-ml polyallomer tube (Beckman Instruments, Inc., Palo Alto, CA).
The gradients were held at 4 C for 4 h. After dialysis to remove the CsCl, the CsCl fractions enriched for competence-conferring activity (the 2.0- and 2.5-ml fractions, Fig. 16) were pooled, and 1.0-ml samples were layered onto the gradients. After centrifugation (143,000 x g, 3 h, 5 C), fractions (1.0 ml) were removed from the bottom of the tube at 5 C, using the apparatus described by Pattee et al. (1968a). The corresponding fractions from all gradients were pooled and immediately assayed for pfu and competence-conferring activity. The results (Fig. 17) showed that the pfu moved to the bottom of the gradient, and no competence-conferring activity could be detected in those fractions that showed the highest number of pfu. The competence-conferring activity migrated much more slowly under these conditions; virtually all of the competence-conferring activity remained in the top fractions (6.0-10.0 ml from the bottom). Only 60% of the pfu were recovered from this gradient, but 109% of the competence-conferring activity was recovered.

To confirm the phage-origin of the competence-conferring activity in the upper fractions, the material from the 8.0-ml fraction was preincubated with 10% serological group B antiserum at 35 C for 30 min prior to being exposed to the cells. This treatment resulted in a complete inhibition of the competence-conferring activity of this fraction.

Effect of Normal Serum on the Enhancement of Transfection Frequencies

It was observed early in this investigation that 10% normal rabbit serum greatly enhanced the frequency of transformation when certain serological group B phages were used to confer competence (Table 7).
Fig. 17. Separation of competence-conferring activity from pfu by rate-zonal centrifugation through a Ficoll 400 (5-25%) gradient.

Fractions were assayed for pfu (triangles) and used to confer competence to 8325thy-101 thrB106 ilv-129 tmm-3110 83A§ pig⁺. Competence was measured by transformation with a saturating concentration of 8325nov-142 DNA. Frequency of transformation (circles) is expressed as the number of Nov⁺ transformants recovered/number of viable cells exposed to the fraction.
FREQUENCY OF TRANSFORMATION $\times 10^{-8}$

ML FROM BOTTOM OF TUBE

PFU PER 0.5 ML $\times 10^2$
For this reason, 10% normal rabbit serum was used routinely in most procedures involving phage-conferred competence. It was also observed that normal rabbit serum could also enhance transfection (and transformation) frequencies in \( \Phi 11 \)-lysogens if the cells were exposed to the serum prior to undergoing the normal transfection (or transformation) protocol.

These observations prompted an investigation into the effect normal serum had on the phenomena of transfection and transformation in \( S. \) aureus.

Because of the ease of manipulation, the system selected to observe these serum-associated effects was the transfection of 8325nov-142, using 80\( \alpha \) DNA. The standard procedure involved TSB cultures that had reached the early log phase of growth (O.D. = 0.20). The culture was divided into samples and appropriate amounts of normal serum were added to each sample which was then incubated at 35 C with gentle shaking for 5 min. The cells were removed by centrifugation (3,400 \( \times \) g, 7 min, room temperature) and subjected to the normal transfection protocol.

Survey of sera Because normal rabbit serum was effective in enhancing the frequency of transfection, sera from various animals were tested to determine the phylogenetic distribution of this phenomenon. Each serum sample (10%, v/v) was added to a TSB culture of 8325nov-142. After 5 min of incubation, the cells were harvested and transfected.

Table 15 is a compilation of the abilities of these sera to enhance the frequency of transfection. Although the sera varied in their ability to enhance, all increased the frequency of transfection by at least 67%.
Table 15. Survey of sera from various animal sources for the ability to enhance the frequency of transfection of 8325nov-142 with 80α DNA

<table>
<thead>
<tr>
<th>Animal Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Obtained from:</th>
<th>Percent Enhancement over Control&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Granite Diagnostics, Inc., Burlington, NC</td>
<td>336</td>
</tr>
<tr>
<td>Cow</td>
<td>College of Veterinary Medicine, Iowa State University</td>
<td>163</td>
</tr>
<tr>
<td>Goat</td>
<td>College of Veterinary Medicine, Iowa State University</td>
<td>67</td>
</tr>
<tr>
<td>Sheep</td>
<td>College of Veterinary Medicine, Iowa State University</td>
<td>142</td>
</tr>
<tr>
<td>Horse</td>
<td>College of Veterinary Medicine, Iowa State University</td>
<td>445</td>
</tr>
<tr>
<td>Human</td>
<td>John Bush</td>
<td>627</td>
</tr>
<tr>
<td>Chicken</td>
<td>T. F. Simpson, Iowa State University</td>
<td>151</td>
</tr>
<tr>
<td>Rainbow Trout</td>
<td>Great Lakes Fish Disease Control Center, Genoa, WI</td>
<td>291</td>
</tr>
<tr>
<td>Fetal Calf</td>
<td>Gibco Diagnostic, Madison, WI</td>
<td>233</td>
</tr>
</tbody>
</table>

<sup>a</sup>10% (v/v) serum used in all assays.

<sup>b</sup>\[\text{Test frequency} - \text{Control frequency}\] x 100

\[\text{Control frequency}\]
However, because of its availability, normal rabbit serum was used in most procedures.

**Serum concentration** Although 10% normal rabbit serum was routinely used, this value was arbitrarily determined from the controls used during neutralization of phage during the phage-conferred competence regimen. In order to determine the actual amount of serum needed to achieve maximum enhancement, samples of a TSB culture of 8325nov-142 were incubated for 5 min with varying concentrations of normal rabbit serum. The results (Fig. 18) showed that only about 1% serum was needed to achieve saturation, and as little as 0.1% resulted in significant enhancement. Concentrations of normal rabbit serum greater than 10% did not result in further enhancement; however, concentrations greater than 50% showed a slight decrease in enhancement (data not shown).

**Duration of enhancement** It became apparent from the experiment in which competence in 8325nov-142 was inhibited by φ11-antiserum that the enhancement of transfection frequencies with normal rabbit serum was nearly instantaneous (Fig. 8). However, in that experiment, the culture was continuously supplemented with fresh serum because of the low neutralizing ability of the antiserum. Therefore, in order to determine the duration of the enhancement of a saturating concentration of serum, a TSB culture was supplemented with 1.2% normal rabbit serum. Samples were removed at intervals and assayed for the ability to undergo transfection. The results (Fig. 19) showed that the addition of 1.2% normal rabbit serum resulted in an immediate enhancement of transfection
Fig. 18. The effect of serum concentration on the enhancement of transfection frequencies in 8325nov-142.

After 5 min of exposure to the serum, the cells were transfected with a saturating concentration of 80α DNA. Frequency of transfection is expressed as the number of 80α plaques observed/number of viable cells exposed to the DNA.
Fig. 19. The duration of the ability of a saturating concentration of normal rabbit serum to enhance the frequency of transfection in 8325nov-142.

Identical TSB cultures of early log phase cells received either 1.2% normal rabbit serum (open circles) or an equal amount of TSB (closed circles) at time "0". Cells were removed at intervals and transfected with a saturating concentration of 80α DNA. Frequency of transfection is expressed as the number of 80α plaques observed/number of viable cells exposed to the DNA.
frequencies, and this enhancement was sustained at high levels for at least 20 min. After 20 min, the enhancement decreased somewhat, but the frequencies did not return to the level of the control by 60 min of exposure.

Attempts to identify the serum component responsible for enhancement
Because of the extreme complexity of the serum, the identification of the component(s) responsible for the enhancement of transfection frequencies would have been a formidable task. However, the following procedures were used to limit the possibilities.

Inactivation of complement In order to ascertain if complement played a role in serum enhancement, normal rabbit serum was heated to 56°C for 30 min to inactivate the complement. When this serum was used in a final concentration of 10%, an enhancement of transfection frequencies of 176% resulted.

(NH₄)₂SO₄-precipitation Normal rabbit serum was precipitated three times with 33% (NH₄)₂SO₄, and the final precipitate was dissolved in borate-buffered saline. When this serum fraction was used in a final concentration of 10%, an enhancement of transfection frequencies of 156% resulted.

Pronase treatment Because the enhancing component of the serum precipitated with 33% (NH₄)₂SO₄, it was suspected that the component(s) was proteinaceous. Therefore, an attempt was made to inactivate the enhancing component(s) with Pronase. Normal rabbit serum was pre-incubated with 1000 µg Pronase per ml at 35°C for 100 min and then used
in an attempt to enhance the frequencies of transfection. The Pronase-treated serum showed no enhancement.

**Analysis of commercially prepared serum fractions** Using $(\text{NH}_4)_2\text{SO}_4$ precipitation, the enhancing component(s) coprecipitated with the gamma-globulin-containing fraction. Therefore, it was suspected that the serum enhancement might be due to a nonspecific binding of the gamma-globulins to the Protein A of the staphylococcal cell wall (Forsgren and Sjoquist, 1967). However, the $(\text{NH}_4)_2\text{SO}_4$-precipitated fraction probably contained proteins other than the gamma-globulins. Therefore, various purified normal rabbit serum fractions were obtained from ICN Pharmaceuticals, Inc., Cleveland, OH. The fractions were dissolved in borate-buffered saline (pH 8.5) by incubating at 35°C for 1 h. Each fraction was dissolved at a concentration equivalent to that found in normal rabbit serum and then tested for enhancing ability by adding 2% (v/v) of the solutions to TSB cultures of 8325nov-142. After 5 min of exposure at 35°C, the cells were harvested and transfected. Table 16 is a compilation of the enhancing activities of these serum fractions. The greatest enhancement was seen with the beta-globulin fraction, although the alpha-globulin fraction also enhanced significantly. No enhancement was observed with either the gamma-globulin fraction or the albumin fraction.
Table 16. The ability of commercially prepared normal rabbit serum fractions to enhance the frequency of transfecting 8325nov-142 with 80α DNA

<table>
<thead>
<tr>
<th>Serum Fractiona</th>
<th>mg/ml in Normal Rabbit Serumb</th>
<th>Frequency of Transfectionc</th>
<th>Percent Enhancementd</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% borate-buffered saline (Control)</td>
<td>--</td>
<td>$8.5 \times 10^{-5}$</td>
<td>0</td>
</tr>
<tr>
<td>2% Normal Rabbit serum</td>
<td>--</td>
<td>$5.9 \times 10^{-4}$</td>
<td>594</td>
</tr>
<tr>
<td>Alpha-globulin (Cohn Fraction IV-I)</td>
<td>7.4</td>
<td>$1.3 \times 10^{-4}$</td>
<td>53</td>
</tr>
<tr>
<td>Beta-globulin (Cohn Fraction III)</td>
<td>7.4</td>
<td>$2.7 \times 10^{-4}$</td>
<td>217</td>
</tr>
<tr>
<td>Gamma-globulin (Cohn Fraction II)</td>
<td>10.0</td>
<td>$6.6 \times 10^{-5}$</td>
<td>-22</td>
</tr>
<tr>
<td>Albumin (Cohn Fraction V)</td>
<td>34.2</td>
<td>$7.3 \times 10^{-5}$</td>
<td>-14</td>
</tr>
</tbody>
</table>

a All fractions prepared according to Cohn et al. (1946), except for the gamma-globulin fraction which was further purified by electrophoresis and the beta fraction which was prepared by a modification of Cohn's procedure (Strong, 1948).

b Calculated from the percentages set forth for rabbit serum in Hebert et al. (1972) based upon 60 mg protein/ml in rabbit serum (Nairn, 1976). Hebert et al. designated alpha- and beta- globulins as comprising 25% of the rabbit serum protein. Therefore, for this study the amount of alpha-globulins was considered to be equal to the amount of beta-globulins in rabbit serum based upon similar proportions in other animals (Rice, 1968).

c Number of transfectants recovered/number of cells exposed to DNA.

d $\frac{\text{Test frequency} - \text{Control frequency}}{\text{Control frequency}} \times 100$

e A negative value indicates that the control frequency was larger than the test frequency.
DISCUSSION

The demonstration of a reproducible method to transform S. aureus (Lindberg et al., 1972) was a significant contribution toward an understanding of the genetics of this important bacterium. Several investigators have used this technique to investigate chromosomal (Sjostrom et al., 1975; Pattee and Neveln, 1975; Pattee, 1976; Kuhl et al., 1978) and plasmid (Lindberg and Novick, 1973; Stiffler et al., 1974) determinants and interactions between genophores (Pattee et al., 1977; Novick et al., 1979) in S. aureus 8325.

Two salient features of the expression of competence in S. aureus are (1) the requirement for a high concentration of Ca$^{2+}$ and (2) the presence of a serological group B phage in either the prophage or vegetative state. The requirement for a high concentration of Ca$^{2+}$ is shared with the expression of competence in E. coli and related Gram-negative bacteria. However, the requirement for a phage appears to be unique to the S. aureus system.

Sjöstrom and Philipson (1974) have attributed the ability of the prophage φ11 (and the closely related phages 83A and φ14) to confer competence to the production of an intracellular early-acting gene product of the phage. This study presents evidence refuting their early gene theory. The data presented demonstrate that the mechanism by which the serological group B phages confer competence is to provide unassembled phage components that are liberated into the medium, presumably by prophage induction, during the normal growth of a
lysogenic bacterium; alternatively, these components can be introduced experimentally in the form of a phage lysate. These phage components adsorb to the surface of the cells and facilitate the uptake of exogenously added DNA when the cells are exposed to a high concentration of Ca\(^{2+}\). Several lines of evidence support this conclusion.

The growth medium from \(\phi\)11-lysogens can confer competence to cells that are not lysogenic for a serological group B phage and, therefore, cannot become competent during normal growth (Sjöström et al., 1973; Table 4, this study). This transfer of competence with a growth medium is suggestive of the production of an extracellular competence factor similar to the ones observed in \textit{S. pneumoniae} (Tomasz and Hotchkiss, 1964) and the group H streptococci (Pakula and Walczak, 1963). However, Sjöström et al. (1973) attributed this ability to transfer competence to infectious phage particles present in the medium that infected the nonlysogenic phage bacteria. In this study the filtered growth media from 8325-4(\(\phi\)11) and 8325\textsuperscript{nov-142} were compared and found to be nearly equal in competence-conferring activity (Table 4). However, the 8325\textsuperscript{nov-142} growth medium was found to contain only about 10 infectious \(\phi\)11 particles per ml (Table 5), an insufficient number to account for the competence-conferring activity observed. In addition, the competence-conferring activity of the 8325\textsuperscript{nov-142} growth medium could be inhibited by treatment with antiserum directed against purified \(\phi\)11 particles (Table 6); therefore, the competence-conferring activity appears to be due to a \(\phi\)11 virion component present in the medium. The predominance of \(\phi\)12 particles in the 8325\textsuperscript{nov-142} growth
medium was unexpected, but may reflect the efficiency with which different phages can initiate a lytic cycle after prophage induction of a multiple lysogen.

The rate and duration of the expression of competence by non-competent cells when exposed to the 8325nov-142 growth medium also suggests that the competence-conferring activity of this medium is not due to infectious particles (Fig. 2). The acquisition of competence observed in this medium was instantaneous; it does not seem possible that even an early phage gene could be expressed in so short a period of time. The time required to reach a maximum level of competence in the population was probably a reflection of the time required for the inoculum to completely adsorb the phage components from the medium and not a function of the increased ability of each cell to bind and take up DNA as it became more competent. The cells remained competent for about 2 generations; the parameters that dictated this period of expression of competence are not immediately apparent. Removing the cells from the influence of the medium did not significantly change the duration of the expression of competence (Fig. 4). Because competence conferred by the 8325nov-142 growth medium did not result in lysogenization by φ11, and because there was no propagation of phage on the cells, the φ11 components cannot be restored to the medium and competence was abruptly lost.

By comparison with the pattern of competence observed in the 8325nov-142 growth medium, noncompetent cells exposed to the 8325-4(φ11) growth medium exhibited a very different pattern (Fig. 3). Although
these cells also showed an instantaneous acquisition of competence, the population continued to express competence for an indefinite period of time. Many of these competent cells did become lysogenized by \( \phi 11 \); however, the extended competence was more likely because of propagation of \( \phi 11 \) on the nonlysogenic cells which restored the \( \phi 11 \) components to the medium. In addition, the competence-conferring activity of this medium was more susceptible than was the content of infectious phage particles to inactivation by Pronase. Thus, it appears that the competence-conferring activity of the 8325-4(\( \phi 11 \)) growth medium is due entirely to \( \phi 11 \) virion components with no contribution from the infectious phage present.

In order to establish that the \( \phi 11 \)-lysogens also become competent by adsorbing \( \phi 11 \) virion components from the medium, it was necessary to inhibit this process without disrupting the metabolic processes of the cells. Theoretically, it should have been possible to completely inhibit the adsorption of the \( \phi 11 \)-components by incubating the cells in the presence of \( \phi 11 \)-antiserum. However, the enhancement of transfection frequencies by serum complicated these experiments. As seen in Fig. 8, the frequencies of transfection were immediately enhanced when either normal serum or immune serum was added to the cultures. However, after 40 min of exposure to the serum, the level of competence exhibited by the cells treated with the \( \phi 11 \)-antiserum began to decrease. This 40 min delay was probably a function of the time it took for the cells that had already adsorbed phage components to lose their competence; in addition, because the low neutralizing capacity of the
serum dictated that fresh serum be continuously added, it is likely that it took 40 min to accumulate an effective concentration of antibodies in the medium. The effect of simply incubating the cells for an extended period of time in a medium that initially contained a high concentration of immune serum was not determined.

The effect of SDS on the expression of competence is somewhat questionable. Although 8325nov-142 cells treated with 0.004% SDS did show a decrease in the ability to undergo transfection, the specificity of this inhibition cannot be clearly established.

The expression of competence by a φll-lysogen was inhibited completely by treating the cells with a high concentration of Pronase (Fig. 10). In addition, treating 8325-4(φ12) cells that had been rendered competent by exposure to 8325nov-142 growth medium with Pronase also inhibited the expression of competence. Thus, it seems that the Pronase can digest the φll-component from the cell surface, rendering it noncompetent. Treatment of 8325-4(φ12) cells with Pronase prior to exposure to the 8325nov-142 growth medium did not inhibit the expression of competence. Therefore, it can be concluded that the Pronase treatment did not destroy any other proteinaceous sites on the cell surface necessary for the binding and uptake of DNA other than those derived from exposure to the 8325nov-142 growth medium. The concentrations of Pronase used in these experiments were considerably higher than the concentrations used in other studies. However, the Pronase used in this study was preincubated at 35 C for 30 min before use to insure that all DNase activity had been destroyed. This
precautionary measure probably also resulted in the loss of some proteolytic activity. In addition, the cell density that was used in these studies was rather low. Therefore, at low enzyme concentrations interactions with the substrate were probably minimal.

This study also demonstrated that a variety of staphylococcal phages can confer competence to cells that normally do not become competent (Table 7). These phages differ greatly in their lytic spectra and, with one exception, all belong to the serological group B. Thus, these phages all have a similar virion structure. Sjöström and Philipson (1974) could not detect significant levels of competence in 8325-4 after it was lysogenized by phages 29, 52, 52A, 79, 53, or 80α. Clearly, these phages can confer competence to 8325-4 when used in the form of a phage lysate.

Although it seems unlikely, it is possible that all of these phages possess a gene similar to the early-acting gene proposed by Sjöström and Philipson (1974) for φ11. In addition, since each staphylococcal typing phage is probably not a pure phage population, it is possible, although unlikely, that all of the serological group B phages are contaminated with a phage similar to φ11. However, phage purified from an 80α lysate could not be shown to confer competence to 8325-4 while the crude lysate and the centrifuged lysate could confer high levels of competence (Table 13). Because all serological group B phages have a similar density (Dowell and Rosenblum, 1962), a contaminating phage similar to φ11 would remain with the 80α particles during purification. Because similar results were obtained with purified
phage 55 (data not presented), it appears that purified phage particles (and, therefore, early phage genes) cannot confer competence. The low level of competence observed when purified phage particles were used to confer competence was probably due to virion components from the lysate that have aggregated with the phage particles.

Moreover, inactivation with UV-irradiation did not significantly affect the competence-conferring activity of the phage 55 lysate (Fig. 12). Even if this hypothetical early gene is extremely resistant to UV-inactivation, it seems improbable that some damage would not have been incurred when the pfu were reduced by 99.999%. In addition, UV-inactivation had no significant effect on the optimum MOI (Fig. 13). Therefore, it does not seem possible that an early gene product is involved with conferring competence. Although Sjöström and Philipson (1974) reported an increase in the competence-conferring activity of φ11 as it was inactivated by UV-irradiation, the authors did not discuss the significance of this observation. It is probable that this increase was due to a decrease in the lytic capabilities of the phage, thus increasing the probability of recovering transformed cells that had been rendered competent by φ11-components present in the phage preparation.

In view of the fact that purified 80a particles could not confer competence, the relative efficiencies of each of the phages in Table 7 in conferring competence to 8325-4 was probably a reflection of the numbers of unassembled phage components present in that particular
lysate. During the course of this investigation it was observed that different lysates of the same phage species could differ in competence-conferring activity although they contained comparable numbers of pfu. A slight variation in cultural conditions during phage propagation might favor the release of unassembled phage components from the host cells. For example, a slight variation in the incubation temperature might increase the activity of the phage lytic enzyme or cause unfavorable alterations in the configuration of the proteins involved with critical steps in the assembly process.

The inability of Sjöström and Philipson (1974) to demonstrate competence in cells lysogenized by phages 29, 52, 52A, 79, 53, and 80a might have been due to the specific cultural conditions used by these investigators. Their cultural conditions may not have favored inefficient assembly of the phage particles after prophage induction, or they may not have favored prophage induction at all. Whatever the underlying reason, it is apparent that the inability of Sjöström and Philipson (1974) to demonstrate competence in these lysogens was due to a dearth of free phage components in the medium or to the presence of an inhibitor of phage adsorption. Similarly, the inability of tsΦ1131 to confer competence to the host cells at the nonpermissive temperature was probably because of the inability of this mutant to initiate a lytic cycle at that temperature.

The competence-conferring ability of the serological group B phages is probably due to a unique property of the unassembled virion...
components that is common to all of these phages. However, one serological group A phage (phage 3A) also demonstrated competence-conferring ability, and this activity was not neutralized by serological group B antiserum. Therefore, it is possible that production of competence-conferring components is simply a reflection of the efficiency of assembly under specific conditions. If propagated under different conditions, phages belonging to other serological groups might produce virion components capable of conferring competence. Nevertheless, it is not possible to ascertain from the currently available data if the cultural conditions used in this study favored inefficient assembly or if this is an inherent property of the serological group B phages and phage 3A.

All of the propagating strains for the reference typing phages, with the exception of Ps 187thy-139, could demonstrate competence after exposure to phage lysates (Table 8). Several of these strains demonstrated competence without experimental preexposure to phage. It is probable that these strains carry prophages whose unassembled components are liberated into the medium and can confer competence by adsorbing to the cells and facilitating the uptake of DNA upon exposure to Ca\(^{2+}\). None of these strains was investigated to elucidate the parameters that determine competence.

Although not particularly germane to the overall purpose of this investigation, the demonstration that many strains of S. aureus can become competent upon exposure to a particular phage was one of the
more significant findings of this study. Studies are already underway to examine the genetics of exfoliative toxin-producing strains of *S. aureus* by using phage conferred competence (Shoham et al., 1978; Martin et al., 1978).

The realization that purified infectious phage particles cannot confer competence requires a reevaluation of the optimum MOI needed to confer a maximum level of competence. It was originally suspected that an MOI of approximately 5 to 10 was needed to attain a maximum level of competence in a population (Fig. 5). It was thought that the adsorption of several serological group B phages resulted in a distortion of the cell surface, thus facilitating the uptake of DNA (Thompson and Pattee, 1977). Although it is now apparent that this model is not entirely correct, the number of virion components that must adsorb to a cell to render it competent has not been determined. The obvious reason for this lack of knowledge is that the only way that the competence-conferring component was identified in this study was by its competence-conferring activity. However, this problem could have been approached by assays using serum blocking power. It is suggested that this technique not be used until the competence-conferring component has been highly purified; it seems likely that other components might be present in a crude preparation that could not confer competence, but could react with phage-specific antibodies.

The fact that cells harvested from early log phase were more responsive to phage-conferred competence than cells harvested from later
in the growth cycle (Fig. 6) was unexpected. During the entire course of this investigation, the peak of competence in early log phase reported by Sjöström et al. (1973) and Rudin et al. (1974) was never observed with \( \phi ll \)-lysogens. In fact, the level of competence demonstrated by \( \phi ll \)-lysogens remained high well into late log phase (data not presented). A possibility is that the phage receptors might be lost during the later phases of growth in the nonlysogen. Ellwood and Tempest (1969) showed that, like \textit{B. subtilis}, the teichoic acid in the cell wall of \textit{S. aureus} was substituted with teichuronic acid if the phosphate ion concentration in the medium was depleted. Because the teichoic acid forms part of the phage receptor site in \textit{S. aureus} (Lindberg, 1973) this substitution might result in the inability of the phage components to adsorb to the cells. In the case of cells that carry one or more prophages, the continuous lysis of a few cells due to prophage induction might restore enough phosphate ions to the medium to meet the requirements of the cells until late log phase.

The inability to generate competence-conferring activity from intact phage particles suggests that the natural assembly process creates a component whose structure cannot be recreated with ease by physical disruption of the virion. The inability to identify a phage mutant that produces an increased amount of competence-conferring component under nonpermissive conditions was unfortunate because of the formidable task of trying to isolate the competence-conferring component from wild-type phage lysates.
This study presents some preliminary steps in the isolation of the competence-conferring component which, hopefully, will lead to the eventual identification of this component. In addition, these isolation procedures were the final demonstration that competence-conferring activity and infectious phage particles (and thus early phage genes) are, indeed, separate entities. Therefore, contamination of the preparation with inactivated phage particles was highly undesirable. Because of its unique stability, phage 80a was selected for these experiments.

The concentration and separation of the proteinaceous material in the phage lysates by bouyant density centrifugation was an immensely useful procedure. Unfortunately, it was not possible to eliminate all of the infectious phage particles from the fractions enriched for competence-conferring activity (Fig. 16). It is doubtful that the phage particles were adhering to the walls of the centrifuge tube because coating the tubes with a 1% solution of bovine serum albumin had no effect on the separation (data not presented). It is suspected that the phage formed aggregates with other material in the lysates (cell membrane and wall components) which resulted in a change in bouyant density.

Concentration of the competence-conferring component in CsCl generally yielded an enriched fraction with high competence-conferring activity. The highest frequency of transformation obtained by this method was $3.7 \times 10^{-4}$. A ten-fold dilution of the fraction reduced the competence-conferring activity of the fraction by about the same
magnitude. This indicates that neither the DNA concentration nor some metabolic property of the cells was the limiting factor in this particular experiment. Therefore, it might be possible to achieve frequencies approaching 1.0 (100%) if the required amount of competence-conferring component is present.

Complete separation of the competence-conferring activity from the infectious phage particles was finally accomplished by rate-zonal centrifugation through a Ficoll gradient (Fig. 17). While the competence-conferring activity appeared to be more stable than the infectious phage particles in Ficoll, neither were especially stable, and the fractions had to be assayed immediately in order to detect the various activities in all fractions. Because the infectious phage particles migrated farther in the gradient, the increased concentration of Ficoll in these fractions may have contributed to their instability relative to that of the competence-conferring activity. The Ficoll did not appear to interfere with assays of either the competence-conferring activity or the phage infectivity when added to a phage lysate immediately before it was assayed (data not presented). Unless a method is found to either remove the Ficoll or stabilize the competence-conferring activity, this technique will have limited use in purifying and identifying the competence-conferring component. The use of sucrose in place of Ficoll in this procedure was not feasible because the activities were even less stable in sucrose (data not presented).
At this point it is very difficult to even speculate on the identity of the competence-conferring component. Because it is neutralizable with antiserum directed against intact phage particles, it might very well be composed, at least in part, of the attachment organelle of the phage. It is possible that it is simply an empty phage virion.

Upon further purification of the competence-conferring component, it should be possible to determine the mechanism by which the phage component facilitates the uptake of DNA. It is not unreasonable, at this point, to suggest that this process might be somewhat similar to the mechanism used by the intact phage during a conventional phage infection to achieve penetration of the phage nucleic acid. The competence-conferring component might be a "pilot protein" similar to the gene H spike protein of ϕX174 that appears to pilot the DNA into E. coli during a normal infection and can also function in the same capacity during transfection (Jazwinski et al., 1975).

Like the E. coli system, the role that Ca\textsuperscript{2+} plays in the induction of competence in S. aureus is not known. Although Ca\textsuperscript{2+} is required for the adsorption and/or penetration of the DNA in the infection process of many staphylococcal phages (Rountree, 1955), the concentration of Ca\textsuperscript{2+} needed for phage infection is very low compared to that needed for the induction of competence. However, it is possible that some of the Ca\textsuperscript{2+} is needed for penetration of the DNA, but additional Ca\textsuperscript{2+} is needed to maintain the DNA in a specific configuration by reaction with the phosphate groups. This specific configuration might be maintained
by the phage virion during a normal phage infection. In addition, it is possible that the Ca\textsuperscript{2+} is needed to initiate a phase change in the membrane phospholipids as described for \textit{E. coli}. However, another possibility is that the Ca\textsuperscript{2+} is necessary to allow the DNA to penetrate beyond the thick peptidoglycan layer of the cell wall. The Gram-positive cell wall has been shown to function in an ion-exchange capacity (Cutinelli and Galdiero, 1967). This ion-exchange capacity appears to be due to the teichoic acid (Heptinstall et al., 1970). It also has been reported that the adsorption of electrolytes to isolated cell walls of \textit{S. aureus} results in an expansion of the cell wall as measured by volume changes (Ou and Marquis, 1970). Although the competence-conferring component might provide the DNA with an attachment site on the cell surface, it might not be able to execute the transfer of DNA across the cell wall without a configurational change induced by Ca\textsuperscript{2+}. Finally, the function of Ca\textsuperscript{2+} in the induction of competence in \textit{S. aureus} might be multifaceted, involving some or all of the mentioned effects and perhaps more.

The ability of serum to enhance the frequencies of transfection (and transformation) was unexpected. This phenomenon has also been observed in the \textit{S. pneumoniae} and the group \textit{H} streptococci transformation systems. However, in both of these systems, at least part of the enhancement can be provided by the addition of serum albumin (Pakula, 1965a; Hotchkiss and Ephrussi-Taylor, 1951). Rabbit serum albumin could not be shown to enhance the frequencies in \textit{S. aureus} (Table 16). The rapidity of the response to serum (Figs. 8 and 9) suggests that this is
probably a surface phenomenon. In addition, a very low concentration (1.0%) is sufficient to result in maximum enhancement (Fig. 18). Pakula (1965a) was able to detect significant levels of enhancement with a concentration of 0.5% in the group H streptococci. The serum component responsible probably is not readily metabolized by the cells, because a low concentration of serum can enhance the frequencies for an extended period of time (Fig. 19). Because all serum fractions do not enhance (Table 16) this stimulation cannot be attributed to a generalized protein effect. The inability of the gamma-globulin fraction to enhance was surprising in view of the nonspecific reaction of gamma-globulins with the staphylococcal protein A (Forsgren and Sjoquist, 1967). Strain 8325 has been reported to contain a very low level of protein A (Nordstrom and Forsgren, 1974); however, certain genetic derivatives of strain 8325 appear to produce copious amounts of this protein (P. A. Pattee, Iowa State University, Ames, Iowa, personal communication). The particular derivatives of 8325 used in this study have not been examined for their content of protein A.

The enhancing factor(s) seems to be contained in the alpha- and beta-globulin fractions. Although it is possible that more than one protein can enhance, it is probable that these commercially prepared serum fractions are not homogenous fractions, but contain contaminating proteins. Because such a low concentration of the factor(s) is needed to observe significant enhancement, a minimum amount of contamination could lead to incorrect assumptions. Therefore, it is not possible to ascertain from these data to which globulin class the enhancing factor
actually belongs. Whatever the nature of this factor, it has been phylogenetically conserved; sera from many diverse species of animals have the ability to enhance (Table 15).

The mechanism by which the serum component enhances the frequencies of transfection is not known; at this point it is even difficult to speculate on this subject. It does not appear to increase the binding of phage components to the cell because the addition of serum before or after exposure to the phage components results in equal enhancement (data not presented). Thus, it appears that the serum does not enhance the level of competence of the cells, but it does enhance the uptake of DNA. The serum component might be a highly basic protein that helps to bind the DNA molecules to the cell surface.
SUMMARY

In order for a bacterium to take up exogenous DNA from the environment it must achieve a specific physiological state referred to as competence. *Staphylococcus aureus* is unique in requiring the presence of a bacteriophage in either the prophage or vegetative state for the expression of competence. The role of the phage φ11 in conferring competence to strain 8325 has been reported to be due to the production of an intracellular protein coded for by an early gene of the phage (Sjöström and Philipson, 1974).

The study presented in this dissertation demonstrated that the ability to confer competence is not an exclusive property of phage φ11, φ3A and φ14, as had been previously reported; all serological group B phages were shown to be capable of conferring competence when added to the cells in the form of a phage lysate. The data indicate that the mode by which a phage confers competence in the prophage state or when added in the form of a phage lysate are identical, and that an early phage gene is not directly involved in conferring competence to the host cell. The ability of phage to confer competence was demonstrated to be due to a proteinaceous subviral component that can arise by either prophage induction or by lytic propagation. This phage component adsorbs to the cells in the culture and facilitates the uptake of DNA upon exposure to Ca$^{2+}$. Observations consistent with these conclusions include:
1. Purified infectious phage particles are incapable of conferring competence.

2. Inactivation of phage with UV-irradiation has no effect on the competence-conferring activity of a phage lysate. In addition, UV-inactivation does not change the MOI needed to achieve maximum levels of competence in a population of cells.

3. The phage component in a phage lysate responsible for conferring competence is separable from the infectious phage particles.

4. Growth medium from 8325nov-142, a φ11-lysogen, can confer competence to noncompetent cells although there are very few infectious phage φ11 particles in the medium.

5. A culture of 8325nov-142 can be inhibited from expressing competence by incubation in the presence of Pronase. In addition, 8325-4(φ12) cells rendered competent by incubation in 8325nov-142 and 8325-4(φ11) growth media can be rendered noncompetent by exposure to Pronase; no other proteinaceous DNA binding site can be detected except for those obtained by incubation in the 8325nov-142 and 8325-4(φ11) growth media.

6. The competence-conferring activity of the 8325-4(φ11) growth medium and the centrifuged 80α lysate show a greater sensitivity to inactivation by Pronase than do their content of infectious phage particles.

7. The competence conferring activities of growth media, phage lysates, and fractions containing the separated phage component are
all susceptible to neutralization by antiserum directed against serological group B phages.

The demonstration of the proteinaceous phage component that is capable of conferring competence is of particular significance because it represents a \textit{bona fide} competence factor that is of phage origin. In addition, the mechanism by which this phage component facilitates the uptake of DNA is probably similar, and might be identical, to the mechanism by which the phage mediates the penetration of infectious DNA during a normal infection.

All of the propagation strains for the staphylococcal reference typing phages demonstrated competence for transformation when exposed to one of the serological group B phages, with the exception of Ps 187 which demonstrated high levels of competence without experimental pre-exposure to phage lysates. These strains might carry a serological group B phage that supplies unassembled virion components to the medium that adsorb to the cell surfaces and confer competence.

Phage-conferred competence did not result in distortion of the genetic linkages observed in strain 8325; therefore, this technique has made many strains of \textit{S. aureus} amenable to genetic manipulation.

The role of normal serum on the enhancement of transformation and transfection frequencies was also examined. Sera from many species of animals were shown to be capable of enhancement. Very low concentrations of serum (1\%) could yield maximum enhancement, and the rapidity of the response suggests a cell surface phenomenon. The enhancing
factor appears to be one or more proteins of the serum that fractionate
with the alpha- and beta-globulins.


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