EARLY EXTRACELLULAR EVENTS IN SP82G DNA INFECTION OF COMPETENT BACILLUS SUBTILIS

GORDON LEE WILLIAMS

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EARLY EXTRACELLULAR EVENTS IN SP82G DNA INFECTION OF COMPETENT

*Bacillus subtilis*

by

GORDON L. WILLIAMS

B.A. Lehigh University, 1969

A THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
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Doctor of Philosophy

Graduate School
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May, 1972
This thesis has been examined and approved.

D. MacSwain
Thesis director, D.M. Green, Prof. of Genetics and Biochem.

F.K. Hoornbeek, Assoc. Prof. of Genetics

Y.T. Kiang, Asst. Prof. of Genetics

G.L. Klippenstein, Assoc. Prof. of Biochemistry

J.A. Stewart, Asst. Prof. of Biochemistry

May 15, 1972
Date
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ABSTRACT

EARLY EXTRACELLULAR EVENTS IN SP82G DNA INFECTION OF COMPETENT

*Bacillus subtilis*

by

GORDON L. WILLIAMS

Competent *Bacillus subtilis* cells are capable of absorbing extracellular DNA molecules and expressing the new genetic material acquired in this manner (44). Uptake of a single molecule of SP82G bacteriophage DNA is not sufficient to permit establishment of an infective center, due to intracellular inactivation of transfecting DNA (18) however, wild type genes introduced in this manner will recombine with DNA of co-infected mutant SP82G bacteriophage, permitting the wild type genome to be expressed in the progeny of mutant infection events (17) Since SP82G DNA molecules are linear, nonpermuted and uniform in molecular weight (19,5), it is possible to use this process of genetic marker rescue to follow the binding and uptake of individual genetic markers on individual DNA molecules.

Analysis of the effects of deoxyribonuclease I (DNase I), physical shear, and temperature shock on DNA-cell complexes in marker rescue demonstrates that sequential attachment of both ends of SP82G DNA to *B. subtilis* precedes entry of the DNA molecule into the cell, and that each attachment is end specific and time specific. The first attachment involves an initial heat-reversible phase followed by irreversible binding of one specific end of the molecule. Following a latent period, the second end attaches to the cell. Entry of the molecule begins immediately when binding of the second end has occurred,
and the molecule is completely resistant to DNase I within 5 min. after this. Polarity of entry is the reverse of that observed in normal phage injection (5).

The initial reversible binding is released by exposure to heat or cold. Released DNA remains infective, but cells which have received either heat or cold treatment lose competence to bind DNA. Competition studies confirm the existence of two distinct sites of irreversible binding. Binding of DNA to the first site is necessary to activate the second. Activation of the second site does not involve synthesis of new protein.

Light lysozyme treatment or exposure to protease facilities marker rescue slightly, but combined protease and light lysozyme treatment before DNA attachment inhibits marker rescue. Treatment of cells with cyanide also prevents marker rescue at time before entry of the molecule. It is hypothesized that after an initial electrostatic attachment, both ends of the molecule sequentially attach to a membrane associated protein molecule. Entry through the membrane proceeds linearly by a cyanide sensitive transport mechanism. Genetic markers on DNA halved by shearing attach and enter in a manner essentially similar to the entry of whole unsheared DNA.

Antisera directed against single stranded SP82G DNA is shown to facilitate marker rescue. A general facilitation is observed if infecting DNA is pretreated with the antiserum. A second facilitation effect is shown to be marker specific, infectivity of late entering markers is more greatly enhanced than earlier entering markers. The first effect is probably due to removal of competing inactive DNA molecules with overlapped (single stranded) ends, or to electrostatic
effects from interaction with the cell surface. The second effect is probably related in some manner to the entry process, suggesting that denaturation may occur during uptake.
SECTION I

COMPETENCE AND DNA INFECTION IN BACTERIAL CELLS: A REVIEW

A number of very excellent and comprehensive reviews of competence and bacterial transformation have appeared within the last three years (12, 22, 58). Those interested in a broader or more complete treatment are invited to go directly to those several hundred pages. There is little merit in repeating the bulk of that material here, since it already exists in a well organized, coherent, and complete form. The present summary will attempt to stick to essentials; significant general data, specific work most relevant to the *B. subtilis* system, and more recent work not covered by the reviews cited.

Bacterial transformation has been studied for over 30 years, however general acceptance of DNA as sole mediator of the genetic transfer process was not complete until the development of satisfactory techniques of purification and quantitation in the mid 1950's. Competence refers to the physiological state of the bacterial cell which permits it to absorb, and express genetically, isolated DNA molecules encountered in the environment.

Cells are not particular about the origin of the DNA molecules they absorb; however, expression of the new material usually requires that donors and hosts be related in some manner. Other general restrictions are a minimal size requirement and double stranded conformation of the infective DNA. Estimates of the minimum viable molecular weight range from $3.0 \times 10^5$ daltons (16) to $10 \times 10^6$ daltons (12). If bacterial cells absorb bacterial DNA the process is called transformation. If the DNA is of viral origin the process is called transfection.
analogous process has been demonstrated in procaryotic cells by the recent discovery that cultured human cells may be transformed by DNA from bacteriophage lambda (39).

Most work to date, however, involves procaryotic systems and DNA from related bacteria or bacteriophage viruses. Most important among recipient bacteria are *Diplococcus pneumoniae* (pneumococcus), in which the phenomenon was first discovered, and *Bacillus subtilis*. Hemophilus influenzae and a number of other bacteria are also suitable hosts, but since infection details vary with the host, the tendency has been to concentrate on a few systems.

The process of DNA infection, either transformation or transfection, is readily divided into three phases. The first phase involves attainment of the physiological state of competence by host cells. The second phase consists of attachment and entry of the DNA molecule. Finally there is, in transformation and marker rescue, the required step in which recombination unites new DNA with the recipient genome or the rescuing phage pool. Recombination is often required in transfection, although it has been suggested that perhaps not all viral genomes need it (42). Several infecting phage DNA molecules may be necessary for successful infection if the host possesses a mechanism for inactivating foreign DNA (18). Marker rescue (17), a variation of transfection which permits finer genetic analysis than simple transfection, also requires recombination of the infecting DNA with other phage DNA already in the cell.

Historically, the cellular physiology of competence and the intracellular recombination event have received more attention than the attachment and entry of the DNA molecule, since it was felt that
they were of greater general significance (22). In spite of this, Erickson (12) in 1969 notes that:

The competent state remains ill defined and the best all inclusive statement concerning the nature of competence may still be that "it is not unreasonable to suppose that... reversible alterations at specific sites on the surface of... cells can result from enzymatic action, and that these alterations make possible the adsorption or penetration of the specific deoxy-ribonucleic acid" (McCarty et al., 1946).

Competence has traditionally been defined as the ability of cells to bind DNA in a DNase insensitive state (58). Irreversibility in this context means that the binding is such that exposure to extracellular DNase does not prevent the genetic transformation, or transfection, event (14). Much of the problem in defining competence biochemically has resulted from the arbitrary nature of this definition, which ignores the physiology of the competent state and the necessary interrelationship between stages in the transformation process. Fragmentation of the process has produced confusion and the more recent trend has been to seek unifying factors instead.

Justification for separating DNA attachment and uptake from the intracellular recombination events arises from experiments showing that uptake of non-homologous DNA occurs in competent cultures of both pneumococcus (30) and B. subtilis (5) without any phenotypic expression or demonstrable recombination (28). Also, cells that are not competent in transformation tests quite capably undergo genetic recombination with genetic markers introduced by transduction (49). On the other hand, separation of the competent physiology of cells from the binding of DNA is an intellectual distinction. Recent techniques for separating competent from non-competent B. subtilis cells from the same culture
depend on the peculiar physiology of competent cells (8). After separation, only the competent cells are capable of binding DNA. Thus the competent state, the attachment of DNA, and its uptake, are best considered as aspects of the same special cell function rather than individual events.

There is little merit in discussing at length the nutritional requirements for producing competent cells. After a period of growth and multiplication, pneumococcus cells become competent for a period of about 15 min (50). Appearance is synchronous and all cells in the culture are capable of binding DNA. Anagnostopoulos and Spizizen (1) first defined conditions for the transformation of vegetative B. subtilis cells. After growth to saturation in enriched media, cells are diluted into a minimal type media and grown until the end of the logarithmic growth phase. Within limits, most laboratories develop their own details of procedure. Recent indications suggest that much of the elaborate traditional procedure may be unnecessary (6, 7, Gordon Williams, unpublished). Unlike the pneumococcal system, only about 15% of the cells in the culture of B. subtilis become competent (23). Different cells become competent at different times (24), and individual cells may remain competent for more than an hour (38). At any single time, then, the percentage of cells competent in a culture is generally less than 10% (37).

The ability of a given bacterial strain to become competent apparently depends upon genetic factors. Incompetent B. subtilis strains have

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1 The term incompetent is used in this discussion to refer to genetically non-transformable strains, non-competent refers to transformable strains at times during the growth phase when they are not competent.
been isolated as mutants from highly transformable strains (66) and the loss of transformability has been shown to be heritable (42). In pneumococcus, Tomasz (56) has isolated a heritably incompetent strain. Mutant genes causing loss of transformability have been shown to be physically linked and closely related functionally to genes for antibiotic resistance and spore formation in both systems (11, 42). More detailed work, lacking in B. subtilis, has identified one incompetent strain of pneumococcus as having lost the ability to synthesize a protein activator substance, called competence factor (CF) (56).

CF protein can be extracted from competent pneumococcus cultures (60) and can induce competence in non-competent cultures (53) at any early growth stage. Cells which have recently lost the property of competence do not become competent on addition of CF (55). Antiserum produced against competent cells, but not against non-competent cells, can inhibit the attainment of competence or, if added to a competent culture, inhibit the initial attachment of DNA to recipient cells (59). This antiserum does not inhibit competence in B. subtilis or H. influenzae, but does prevent transformation in related streptococcal strains, suggesting that at least specific activator proteins and perhaps even the entire competence mechanism is species specific.

The latter suggestion is supported by the general physiological data. No significant differences are observed between the physiology of competent and non-competent pneumococcus cells (57). In contrast to this, B. subtilis cells are in a state of general biosynthetic latency resembling the physiology of early sporulation (65). Synthesis of cellular polymers is much reduced (34). Competent bacteria are more resistant to penicillin (37) and to thymineless death (3). There is a long
delay, 3 to 5 hrs, in the expression of newly transformed properties
and in the beginning of replication of newly transformed cells (38).
Competent cells are deficient in photoreactivating capacity (39) and seem
to have a fraction (5%) of their DNA in a form resembling single strand-
edness (20). Competent cells are shorter than normal cells by about
5 μ (23) and contain an unusually large number of uninucleate cells,
normal cells being mostly binucleate (45). Many cells in competent
cultures are seen to resemble cells in early stages of sporulation,
and it is to these cells which isotope labeled DNA binds (65). Finally,
and most importantly for this paper, the biosynthetic latency does not
prevent rapid physical (4) and genetic (61) integration of transforming
DNA, and replication of bacteriophage introduced by transfection also
proceeds with nearly normal rate (17).

The question of the elaboration of a specific CF type activator
protein has not been settled in the B. subtilis system. Early experi-
ments located competence specific protein factors which could be re-
moved from competent cultures by washing (1, 9). Removal of these fac-
tors caused immediate loss of competence, and competence was recovered
when the washed supernate was added back to the cells (1), but this
reversibility is sensitive to Actinomycin D and chloramphenicol (27),
even in the presence of saturating amounts of the "CFV" proteins.
Chloramphenicol blocks the appearance of these specific proteins (1)
but does not prevent attachment or uptake of transfecting DNA (27), nor
does treatment of competent B. subtilis cells with extracellular pro-
nase destroy competence (this paper) although it does in the pneumo-
coccus system (56). This suggests that the competence specific extra-
cellular proteins in the B. subtilis system, while of possible peri-
pheral or secondary importance, do not behave as the CF activation protein in pneumococcus, which is a single protein possessing specific functions of primary importance in that system. Erickson (13, 12) has shown that competent _B. subtilis_ cells bind other macromolecules besides DNA. Isotope labeled rabbit gamma globulin, DNase I, and single stranded (denatured) DNA are absorbed irreversibly by competent cells. Competence in _B. subtilis_ is apparently a generalized, diffuse process rather than a highly specific one, making the function of the extracellular proteins, if they have one, difficult to specify.

Considering the potential importance of ionic interactions in binding macromolecules to bacterial cells, little work has been done in this area. Charge density at the cell surface, measured by electrophoretic mobility, has been shown to vary with the physiology of the bacterial culture (40) and alterations of surface charge have been implicated in conjugation in _E. coli_. Jensen and Haas (26) used the ability to penetrate cellulose acetate of varying pore size as a measure of the electrokinetic potential at the cell surface, showing that this fractionates particles as a function of charge rather than size. Comparing results from cultures at varying stages of competence, they concluded that competence was directly related to the surface charge of the recipient cell (25). Surprisingly, competence is characterized by an abrupt increase in electronegativity in individual cells. The negative charge is slowly discharged over a period of one or two hours, about the length of time which the competent state lasts in individual cells. Perhaps this explains in part the generalized binding properties of competent cells mentioned by Erickson (12), but it would be difficult to imagine that this electronegative shift would facilitate
the binding of negatively charged DNA molecules to the cell.

On might suggest that the role of the required Mg$^{++}$ and Ca$^{++}$ ions in transformation is to neutralize, at least locally, this general net negative charge. Teichoic acid residues have been shown to facilitate association of these ions with the cell wall, and teichoic acid residues have been implicated in the binding of CF to competent cells in pneumococcus (57, 60). Other intermediary particles with significant positive charge may facilitate binding by attaching to both the DNA and the cell wall ionically. It is known that the pneumococcal CF has a net positive charge (31). A third possibility for *B. subtilis* is that binding may occur at specific pockets, invaginations, or holes in the cell wall where the negative charge is locally interrupted or reversed. Structural considerations suggest that all three possibilities may be important.

The cell wall of *B. subtilis* is very complex, consisting of a mucopeptide (composed of N-acetyl glucosamine (NAGA), N-acetyl muramic acid (NAMA), alanine, glutamic acid, and diaminopimelic acid), teichoic acid (a polyglycerol phosphate with D-alanine or D-glucosamine on glycerol carbons 1 or 2 and a poly-N-acyl-hexosamine on the terminal phosphate), insoluble protein, and a few minor heteropolymers (44). The mucopeptide is a polymer of repeating units of NAMA and NAGA, with the three amino acids attached as a tripeptide to the NAMA. NAMA and NAGA are linked by (1-6) and (1-4) glycosidic bonds, the latter being susceptible to lysozyme. Cross linking is thought to occur by peptide bond formation between the terminal amino acids of adjacent polymers, linking two NAMA residues together (44). A rigid two or three dimensional net would result. Teichoic acid residues and other heteropolymers confer
antigen specificity, thus some portion of their molecules must be exposed (63); however, the exact mechanism of association is not known. Some of the proteins are apparently present between the cell wall and the membrane and are not exposed.

There are chemical differences between cell wall composition of highly and poorly transformable B. subtilis strains. Highly transformable strains contain more galactosamine than poorly transformable ones (67), and the percentage of galactosamine in the cell wall increases with growth of the culture (65), reaching a maximum at the time of maximum competence. Galactosamine is isolated with the teichoic acid fraction of the cell wall (67), and Young has suggested that an increased substitution of galactosamine on terminal phosphorous groups could decrease the net negative charge of the cell wall to facilitate penetration.

In pneumococcus, choline, a component of teichoic acid, is an essential growth requirement for the development of competent cells. Removal of choline causes abrupt loss of reactivity of cells with CF, and binding of radioactive DNA is inhibited (60). This effect is reversible by readdition of choline. If choline is replaced by structural analogues, bacteria do not become competent and their cell wall is completely resistant to the action of isolated pneumococcal autolytic enzyme (57). The B. subtilis autolytic enzyme, N-acylmuramyl-L-alanine amidase, increases in cells as they approach maximal competence and decreased autolysin activity is observed in incompetent B. subtilis strains (67). Young suggests that the modification of the cell wall by this enzyme might produce focal relaxation and gaps which facilitate the penetration of DNA (63).
Other evidence exists which suggests that DNA may penetrate the cell wall and make attachment to the cell membrane, rather than the wall itself. Bound DNA cannot be stripped off with lysozyme, which removes the cell wall, and autoradiography shows that isotopically labeled DNA remains attached to the protoplasts (65). Also, DNA does not form complexes with isolated cell wall or cell wall polymers, nor does the addition of cell wall polymers to competent cultures inhibit transformation (65).

Further experiments with lysozyme add details to this argument. Hirokawa and Ikeda (21) reported genetic transformation in protoplasts stabilized in sucrose containing medium. Although cell multiplication did not occur, transformants were scored on the appearance of genetic linkage between donor and recipient molecules in DNA extracted from the protoplasts. Prozorov (41) showed that light lysozyme treatment of competent cells results in a tenfold increase in transformation when treatment immediately precedes attachment of the DNA to the cell. Treatment at times prior to this has no effect; resynthesis may repair the cell wall. Treatment at later times was either inhibitory or had no effect, and larger doses of lysozyme were also inhibitory. Treatment was light enough so that these cells gave rise to colonies on normal agar. A number of detailed studies have confirmed these results (51, 52, 36). Severe lysozyme treatment, although it prevents the formation of transformants, does not release isotopically labeled DNA from the cell (65), and the loss of transformants does not represent cell death, as 90-100% were viable but untransformed on supplemented agar (51). The conclusion is that attachment, but not entry can occur in the absence of cell wall. This does not contradict Hirokawa's
experiments which were done on protoplasts containing some cell wall material.

Autoradiography shows DNA inside the cell wall and bound to the membrane after DNase resistance was achieved (65). Young also noticed an increase in the presence of mesosomes and membrane invaginations in cells with transforming DNA attached to them. Cells with attached labeled DNA were generally in a state resembling early stages of spore formation, and Young hypothesized a relation between abortive sporulation and competence. Indeed, the presence in more than trace amounts of the ions needed to complete spore formation (Fe^{++} and Mn^{++}) inhibits competence in otherwise normal cultures, and other ions do not show this effect (7).

The structure of the membrane-DNA complex has been investigated by a number of methods. Venema et al. (62) reported that transformation could not be detected for the first 20 min after adsorption of molecules to the cell. After 20 min, activity was recovered and linkage between donor and recipient DNA was present. Before the donor marker regained activity it was shown to demonstrate altered heat resistance and renaturation abilities relative to the recipient markers, and it was suggested that this could be due to denaturation of DNA during uptake (61). Erickson (12) presented further data demonstrating single stranded characteristics of adsorbed DNA. Antibodies specific for single stranded DNA were used to inhibit transformation. The antibody did not react with the native DNA and significant inhibition was observed only when recipient cells were incubated with antibody for long periods of time prior to exposure to DNA. Since rabbit gamma globulin may be absorbed into competent cells (12), it was suggested that the
inhibition reaction occurred within the cell boundaries, though it is not made clear whether the term boundary means cell wall or cell membrane.

Further evidence for denaturation of DNA during entry is readily available. In pneumococcus, heteroduplex molecules of infecting DNA may be used to transform. Results show that only one strand of the donor molecule is incorporated into the recipient genome (15). Approximately 50% of infecting isotopically labeled DNA is degraded into fragments while the other half remains undegraded and is incorporated into the genome (15). Conversion to the single stranded state in DNA infected \textit{B. subtilis} is shown by experiments in which infecting DNA is rescued at various times after binding to the cell. At first double stranded segments of DNA are recovered, but as entry proceeds, increasingly larger amounts of single stranded DNA are recovered, and eventually all DNA recovered is single stranded; the balance of the infecting DNA degraded to nucleotides (76).

The denaturation probably occurs when the DNA is in a DNase resistant stage but membrane associated during actual entry. DNase resistant cell-DNA complexes were treated extensively with DNase I, (which Erickson claims may also be absorbed by competent cells over a period of time). Exposure to anti-single stranded DNA antibody after this treatment results in the loss of up to 90% of the expected transformants (12). Strauss (48) has shown that transformant complexes remain cyanide sensitive six minutes after attaining DNase resistance. These treatments, as well as the lysozyme inhibition mentioned earlier, apparently attack the DNA-membrane complex after the molecule is inside the cell wall (and conventionally DNase resistant, although Erickson's results apparently deny that resistance is total) but before entry
through the membrane into the interior of the cell; the state in which Young's autoradiographs show the DNA (65).

Attainment of this DNase resistant state by infecting DNA molecules has been shown to occur in a linear fashion in *B. subtilis* (47) and in a linear polar fashion in pneumococcus (15). Phenethyl alcohol (0.05%), while permitting attachment of transforming DNA to the cell, prevents attainment of the DNase insensitive state by 95% of the attached DNA (43). The inhibitory effects of exposure to heat (35) and to cold and physical shear (10) also involve the initial binding of the molecule before its entry into the DNase resistant state.

Tomasz (55) has presented the following model for transformation in pneumococcus. All steps in the process and all molecular mechanisms and active substances have been identified, so there is little question that the model provides a reasonable outline of the process in that system. He suggests that:

A. At the end of log growth, a few exceptional cells begin to synthesize CF, which is a surface constituent and is also released externally. It reacts with other cells, inducing them to also produce CF. Under optimal conditions all cells can react with the CF.

B. In the presence of DNA, cell associated CF binds with the DNA, and the DNA is rapidly and linearly transported to a DNase insensitive location.

C. Uptake involves an exonuclease which degrades one strand, pulling the sister into the cell interior. Eclipse reflects the single stranded nature of this stage.

D. Inside the cell, donor DNA seeks its homologue. Extracellularly a competence inhibitor appears and inactivates CF.

E. Cells lose competence and recombination produces heterozygous transformants.

Although this model is plausible for *B. subtilis* also, Erickson (12) presents another model which also fits the data. In the light of
demonstrated differences between the two systems, Erickson's mechanism is perhaps more satisfactory than attempts to extrapolate Tomasz's model the *B. subtilis* system. He suggests:

A. As the culture enters the stationary phase of the growth cycle and conditions become unfavorable for growth, a proportion of the population is caught between cell divisions. Since the cells are preparing for sporulation, one genome is complete (the potential spore) while the other is partially replicated. Protein synthesis continues in the absence of significant nucleic acid synthesis, cell wall structure is weakened in the area of cell division and the charge distribution changes appreciably.

B. In the presence of DNA and Mg++ the cells rapidly bind the DNA at the exposed plasma membrane. The DNA becomes linearly resistant to DNase as it is pulled inside the cell wall and bound tightly to the membrane.

C. If the recipient DNA-cell complex is left in the exhausted growth medium, spores are formed and no transformants are found (the potential transformant genome is not the spore genome).

D. If the cell-DNA complex is transferred to fresh media, sporulation is aborted. DNA synthesis resumes and entry can then occur. As time passes, hydrolysis of the membrane associated DNA occurs, the strands become less firmly bound and can be replaced by subsequently added DNA in the displacement reaction. Cell division begins and the cells begin to lose competence.

This model explains adequately the physiological nature of the competence process in *B. subtilis*, and permits the incorporation of a great amount of specific data into its framework. The model might be appended to add a comment about the membrane associated and partially denatured condition of replicating DNA. If the DNA caught in replication were membrane attached in a region close to the attachment of transforming DNA on the other side of the membrane, and if slight denaturation of the membrane attached transforming DNA also occurred, simple transport across the membrane of short single stranded fragments of transforming DNA would bring them together with partially denatured recipient DNA providing an ideal opportunity for recombination. Dubnau et al., (10) have observed membrane bound DNA being progressively converted to small
double stranded fragments and then to small single stranded fragments as entry of the DNA occurred.

The remainder of this work supports and further develops Erickson's hypothesis. In addition to lending credence and a further elaboration of the details of early binding to this framework, these studies make it possible to pinpoint and explain the nature of inherent differences which exist between the observations for transfection and those presented here for marker rescue. The research is presented in the form of three papers.
REFERENCES


10. Dubnau, David, C. Cirigliano (1972). Fate of transforming DNA


19


53. Tomasz, A. (1965a). Control of the competent state in pneumococ-
cus by a hormone-like cell product: An example for a new type of regulatory mechanism in bacteria. *Nature*, 208, 155-159.


63. Young, F.E. (1965). Variation in the chemical composition of the cell walls of *Bacillus subtilis* during growth in different media.


SECTION II
DNA-CELL INTERACTIONS BEFORE DNA ENTRY

Introduction

The kinetics of DNA entry during the transformation of *B. subtilis* cells suggests a linear sequence of entry of linked genetic markers, preceded by a definite lag period before DNase resistance is attained (9, 13). In *Diplococcus pneumoniae* Gabor and Hotchkiss (4) have shown that DNase resistance is attained by the linked genetic markers Sdb in a linear manner; resistance shows a unique direction of entry, in the order S -d -b. The occurrence of a unique polar entry presupposes either i) an uptake process that is not specifically directional, but is operating on a population of molecules that have a unique distribution of these genetic markers relative to the point of uptake on the molecule, as Gabor and Hotchkiss (4) suggest, or ii) a specifically directional uptake process, operating on a population of molecules where the positions of the genetic markers relative to the ends of the molecules are random. To distinguish between these two alternatives is difficult by bacterial transformation, since substantial breakage of the genome (either specific (4) or nonspecific) occurs during the isolation of DNA. The distinction between unidirectional and bidirectional linear uptake should be relatively straightforward if the entry of a genetically nonpermuted DNA can be studied. DNA of *B. subtilis* bacteriophage SP82G bears its genes in a nonpermuted sequence, as shown by shear studies and genetic rescue (6), by the interruption of bacteriophage injection (7), and by $^{32}$P-decay effects on gene transfer (McAllister and Green, in preparation). SP82G phage DNA is readily extracted with phenol or
NaClO₄ to yield a DNA of uniform molecular weight that represents the entire known genome. SP82G DNA infects efficiently by both transfection and marker rescue assays (5, 7).

Use of the transfection system to follow DNA entry is limited by a multimolecular requirement for production of an infective center (7). With marker rescue, however, it is not only possible to focus on the behavior of a single molecule, but on a single genetic marker, making it most useful for the present study.

Our experiments use genetic marker rescue to examine the sensitivity of specific genetic markers to physical shear, DNase I, and temperature shock after adsorption of DNA molecules to cells. The genetic results are consistent with the physical behavior of DNA.
Materials and Methods

**Bacterial Strains;** *B. subtilis* strain SB1 (11) was the host cell.

**Viral Strains;** The bacteriophage used for DNA isolation was wild-type SP82G phage (5). Prior infection for the rescue of markers was performed with three temperature-sensitive double mutants of SP82G; E14-H24, H167-A4, and G55-H177 (8). These mutant strains are constructed (see Fig. 1) of closely linked pairs of mutants representing the right end, middle, and left end of the genetic map, respectively (7). Close double markers are used to prevent the production of wild-type infective centers due to reversion.

**DNA Isolation;** Concentrated preparations of wild-type phage were purified by centrifugation on a CsCl step gradient. DNA was extracted (3) by incubation with NaClO₄. Care was taken to prevent fragmentation by shearing. Analysis of this product by centrifugation in a 5 - 20% sucrose gradient (pH 7.0) showed the product to be uniform in size; it sedimented at a rate just slightly faster than whole SP82G DNA isolated by phenol extraction; (molecular weight = 115 x 10⁶). Carefully prepared DNA extracted with phenol was equally suitable for these experiments.

**Competence and Media;** Competence regimes and media were similar to those of Spizizen (12); they have been described in detail, along with a description of the plating media (5).

**Marker Rescue;** The technique of marker rescue with preinfecting mutant phage has been described (7).

**Sensitivity of Attached DNA to Shear, DNase, Temperature Shock;** Undiluted competent cells were infected for 6 min. with mutant phage. An input ratio of five phage per bacterium assures that most phages are
Figure 1. **Genetic Map of SP82G Bacteriophage Genome.** Map units represent corrected recombination frequencies relative to other markers not shown. The physical and genetic map are colinear and the markers NG14 and NG80 represent the left-most and right-most mapped markers on the molecule respectively. Functionally, G55-H177 represents two early phage functions involved with DNA synthesis; H167-A4, two markers involved in tail synthesis; and E14-H24 two late functions involved with head synthesis. All data courtesy D. Green and D. Laman (in preparation).
adsorbed at the end of the preinfection period. Since only 0.5 - 2% of
the phage remain free in the medium after 6 min under these conditions,
it is unnecessary to treat with phage antibody to remove excess phage.
The infected cells are then briefly exposed to wild-type phage DNA (10
μg/ml). After 15 seconds at this high concentration to allow attach­
ment of DNA, the cells are diluted 200-fold with warm LS medium (5) to
terminate adsorption. Samples were taken over a 25-min period after
dilution and plated at 47° to measure wild-type infective centers.
The number of wild-type infective centers produced under these conditions
remained constant over the 25-min period; hence, no new infective centers
were formed during this interval. DNA is limiting in these experiments,
since undiluted samples plated over a similar 25-min period show an
exponential increase in the production of wild-type infective centers
for the first 15 min. Without DNA, the infected cells give no wild-
type infective centers, since the reversion rate of the close double
mutants used is 10^{-8} or less.

After termination of the adsorption period, the infected cells
were incubated at 33°; 10-ml aliquots were taken at 2 min intervals
for experiments (see below) then assayed at 47° to determine the yield
of wild-type infective centers. Production of a wild-type infective
center reflects the uptake of the wild-type alleles corresponding to
the specific mutant genes of the preinfecting phage. Inability to pro­
duce wild-type infective centers represents some failure of either the
attachment, uptake, or expression of the wild-type DNA in the region of
the molecule corresponding to the mutant markers of the preinfecting
phage. We controlled attachment of the molecules and restricted the
experimental treatment to times before entry of the molecule to avoid
any influence on expression of the wild-type genes intracellularly; thus, our observations should reflect differences in the process of DNA uptake.

Samples were exposed to four sets of conditions; (a) Samples in 1 mM MgCl₂ were incubated with 1 mg/ml of DNase I for 1 min at 37°. (b) Samples were sheared in a Sorvall Omni-Mixer for 30 sec. at 11,500 rpm. (c) Samples, (chill control), were chilled to 4° and held at this temperature for 10 min. (d) Sample controls were plated immediately.

Recovery of Active DNA Released from the Cell Surface; Experiments were done to recover any active DNA that might be released from the cell-DNA complex by shearing. Active genetic markers released by shearing were detected by their ability to be rescued when exposed to fresh cells.

The procedure involves preinfection of competent cells with mutant bacteriophage as described previously. DNA adsorption was carried out at a lower concentration (0.1 ug/ml) and for a 30 sec. period so that unadsorbed DNA from the supernatant would not obscure the activity of DNA subsequently released by shearing. Removal of cell-DNA complexes by centrifugation after adsorption and subsequent assay of the supernatant with fresh preinfected competent cells shows that less than 5% of the originally introduced DNA remains as genetically contributing DNA under these conditions.

The DNA exposure was terminated by 1:5 dilution in 33° LS media. Ten ml portions were removed for shearing at 3 min. intervals for the first 15 min. following dilution. Shearing at 11,500 RPM was shown to fragment the released DNA extensively, so shearing was done at 6,000 RPM for one min. The samples were either sheared immediately or chilled to 4° and held until all samples had been taken before being sheared.
Sheared samples were centrifuged at 5,000 g in a Sorvall RC2-B for 20 minutes to separate the DNA-cell complex from the uncomplexed DNA released into the media by shearing. Six ml of the 5,000 g supernatant was warmed to 33° and added to one ml of fresh undiluted preinfected competent cells. The mixture was incubated at 33° for 20 minutes to allow wild-type markers which had been released by the shearing to infect the fresh cells and form wild-type infective centers.

As a control, the 5,000 g supernatant was plated directly to assure that no wild-type infective centers remained from the first infection event. Also, 1 ml of preinfected competent cells was incubated for 20 minutes at 33° with virgin DNA at a concentration equivalent to that expected in the 6 ml supernatant.

Attachment of Radioactive Labelled DNA: Wild-type SP82G phage were labelled with radioactive $^{32}$P as described by McAllister (10). The phage were then purified on a CsCl step gradient and the DNA extracted (3). Following dialysis, the DNA was analyzed for molecular weight and the extent of radioactive labelling was determined.

Competent host cells were preinfected with mutant phage and exposed to the radioactive DNA (2,000 cts/min/ug) at a concentration of 10 ug/ml for 30 seconds, then diluted 1/20 and incubated at 33°. Twenty five ml portions were taken at 3 min intervals for treatment (see below). They were then centrifuged at 5,000 g for 20 min and the pellet was counted on a Nuclear Chicago gas flow counter to determine the amount of radioactivity attached to the cells.

Samples were exposed to 47° or 4° for 2 min, or to 1 mg/ml DNase I for 1 min at 37° in 1 mM MgCl$_2$, or to 30 sec shearing at 11,500 RPM in the Sorvall Omni-Mixer. Some samples were chilled to 4° before shearing.
Results and Discussion

Little is known about the ends of infecting DNA molecules in transfection or marker rescue. The biological activity of native SP82G DNA coincides with the major DNA peak in CsCl density gradients. This DNA is primarily unbroken and represents nonpermuted phage genomes (6, 10). When sheared to lower molecular weights, SP82G DNA shows loss of linkage only for marker pairs spanning the regions of breakage, not for other marker pairs. Both terminal and central fragments of lightly sheared DNA show marker rescue (6). Controlled treatment with DNase II results in reduction of molecular weight, the unlinking of distant markers and the production of small fragments which are rescuable (Green and Laman, unpublished). Thus, if specific ends are required by infecting DNA molecules, the ends generated by shearing and DNase II are equivalent to natural ends in this respect.

When SP82G DNA is denatured on an alkaline sucrose gradient and reannealed, only the portion of the gradient corresponding to unbroken single strands shows any ability to contribute genetic markers (Fig. 2). Lower molecular weight fractions would be expected, on reannealing, to have various degrees of overlap at one end of the molecule. The total lack of activity in these fractions contrasts with the relatively high activity of shear and DNase II products of similar molecular weight. This suggests that a single overlapped end may be sufficient to prevent DNA uptake.

Experiments were undertaken to examine the physical association of infective SP82G DNA with cells during the extracellular steps in DNA infection. The results of the experiments with isotopically labelled DNA (Table I) show that 47° causes the release of virtually all the label
Figure 2. Activity of Renatured SP82G DNA in Marker Rescue.

SP82G phage DNA (1 ug) labelled with $^{32}$P was overlayered on a 5 ml alkaline sucrose gradient (0.1 M NaOH 0.9 NaCl 5-20% sucrose) and centrifuged in a SW 39 rotor with the Spinco L2-50 centrifuge at 35,000 rpm, 110 minutes at 23°. Following centrifugation the bottom of the tube was pierced and eight drop fractions were collected into 0.1 ml of 0.2 M KH$_2$PO$_4$. Portions of each fraction were either unannealed ($\bigcirc$), or annealed at 60° for four hours ($\bigcirc$). Each fraction was assayed with competent cells pre-infected with the double mutant H167-A4, and counted for radioactivity in a gas flow counter. The position for T7 was determined from parallel sedimentation runs. The molecular weight (1) of the single stranded material which recovers infective activity is 50 x 10$^6$ daltons. Assay of these fractions using cells preinfected with the distantly linked double H177-e119 (not shown) shows an identical pattern.
from the cells before 5 min. By 5 min the nature of the binding changes; after this time no label is released by 47° treatment. A similar release of 50-60% of the label, is observed upon chilling to 4° before 5 min. The initial binding of DNA to the cell is thus sensitive to heat and to a lesser extent to cold. A change in the binding occurs by 5 min, after which cold and heat treatments no longer release the DNA.

Shearing (Table I) releases 65-75% of the isotope label until 12 min. By 15 min all label is insensitive to shearing. Chilling to 4° before shearing increases the released label to 80-85% during the 12 min shear sensitive period. DNA remains in a DNase sensitive state, presumably extracellular for 12 min (Table I) after attachment to the cell. It then becomes progressively resistant to DNase until 17 min, when resistance is complete.

The uptake of all genetic markers is inhibited by 47° before 5 min and by 4° before 10 min (Table II). This is consistent with the observed release of isotope label before 5 min. Cold sensitivity after this time is discussed below. The right end marker, E14-H24 achieves shear resistance by 5 min and DNase resistance between 10 and 12 min (Table IIa). This marker is not recovered in the supernatant when sheared after 4 min (Fig. 4B). Before 4 min, release by shearing is greatly facilitated by prior chilling. Although still DNase sensitive, this marker seems too tightly bound after 4 min to be sheared, suggesting that initial attachment occurs at this end. It also achieves DNase resistance earlier than the other markers, suggesting that it may enter first.

The uptake of the center marker, H167-A4, is inhibited by shear and DNase until after 12 min. By 14 min, this marker has achieved
TABLE I

Experimental Release of DNA from DNA-Cell Complexes

Percentage of applied \([^{32}\text{P}]\) DNA which remains cell associated following treatment as indicated at the given times.

<table>
<thead>
<tr>
<th>Min.</th>
<th>DNase</th>
<th>Shear</th>
<th>Chill Shear</th>
<th>47°</th>
<th>4°</th>
<th>Control</th>
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<td>8.0</td>
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<td>14.0</td>
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<td>5.5</td>
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<td>Min.</td>
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</table>

* Numbers represent the number of wild-type infective centers produced per ml sample when treated as indicated at the given times, then incubated at 47°
Figure 3. **Uptake of Genetic Markers on Whole SP82G DNA.** Data from Table II are presented graphically:

A. Cells exposed to 47° at times after addition of DNA
B. Cells exposed to 4° at times after addition of DNA
C. Cells–DNA complex physically sheared at given times
D. Cells exposed to DNase I at times after addition of DNA

- Cells infected with phage mutant E14–H24
- Cells infected with phage mutant H167–A4
- Cells infected with phage mutant G55–H177.
Figure 4. **Timed Recovery of Genetic Markers Following Release by Shearing.** Wild-type phage DNA was attached to cells and then released by shearing at the times indicated. Released DNA was recovered by centrifuging away the cell associated DNA complexes, and the supernate was used to infect fresh cells.

- **A.** Recovery of genetic marker G55-H177
- **B.** Recovery of genetic markers E14-H24
- **C.** Recovery of genetic marker H167-A4

- Represents samples chilled before shearing
- Represents unchilled shear samples
- Represents the control showing the recovery of markers from unattached (free) DNA under similar conditions.
MINUTES AFTER DNA ADSORPTION

ACTIVE DNA RECOVERED AFTER SHEARING

WILD TYPE IC'S / ML SUPERNATANT
resistance to both conditions (Table IIb). Recovery of the marker in cell supernatant after shearing is enhanced by prior chilling (Fig. 4C). Recovery remains constant for 12 min, then disappears by 15 min. Since the isotope experiments suggest DNA entry occurs between 10 and 17 min, this marker remains sensitive to shear and DNase until its entry, between 12 and 14 min.

The marker G55-H177 represents the left end of the map. Uptake sensitivity to shear disappears with the chill sensitivity, after 10 min, but this marker remains sensitive to DNase for 15 min (Table IIc). About 20% of the marker activity may be recovered from the supernatant following release by shearing. After 10 min the marker is no longer released by shear. Chilling before the shear event increases recovery, as with the other markers, throughout the chill sensitive period. (Fig. 4).

We interpret these data to mean that events prior to the entry of DNA into the cell involve about 15 min after attachment of the molecule. This extracellular time may be subdivided into three periods. The first 3 to 5 min represent a heat sensitive period of reversible binding, involving the end of the molecule represented by the marker E14-H24. During the period between 5 and 12 min, after adsorption, the middle region (H167-A4) and left end (G55-H177) of the molecule are sensitive to release by shearing, but the attached end (E14-H24) is not. Chilling to 4°C does not result in the release of either recoverable genetic markers or 32P label from the cells but it is effective in preventing marker rescue during this time. Since the molecule is still outside the cell, demonstrated by the release of markers and 32P label by shearing and the sensitivity to DNase I of all marker pairs, chilling apparently prevents entry of the DNA without causing it to be
released from the cell or degraded by nuclease activity. It remains attached but uninfective.

As indicated in both Table II and Fig. 4, the center of the molecule remains sensitive to shear longer than either end. In both experiments the two ends become shear insensitive well before attaining DNase resistance. The center of the molecule (H167-A4) remains sensitive to shear, suggesting that it is not tightly bound to the cell until several minutes later, when it simultaneously achieves shear and DNase resistance. This suggests that both ends of the molecule attach to the cell prior to entry. If mandatory attachment of both ends precedes entry, a simple mechanism may be envisioned by which chill prevents uptake without causing release or degradation. Chilling to 4° could prevent binding of the second end by producing an alteration in the cell receptor site. Chill resistance appears simultaneously with the loss of shear sensitivity by the left end marker (G55-H177) in both genetic experiments. We suggest this reflects the binding of the second end. This argument is strengthened by the fact that 4° no longer prevents entry after 10 to 12 min. If the effect of chilling were to inhibit the actual entry process, rather than a second binding reaction as we suggest, it should continue to inhibit effectively until entry has been completed for late entering markers. It does not.

Entry of the molecule occurs between 12 and 15 min. The bulk of the $^{32}$P label achieves DNase resistance between 12 and 15 min. At 12 min, the marker E14-H24 becomes resistant to DNase I. The marker H167-A4 achieves resistance between 12 and 15 min, and the marker G55-H177 becomes resistant after 15 min. This confirms the order of entry in marker rescue suggested by earlier work (S. Westerman and D.
Green, unpublished). The rate of entry is of the same order of magnitude as that observed for phage injection in SP82G (10). The order of marker entry however, is the reverse of that observed in normal phage injection by McAllister (10).

In summary, these experiments suggest the following stages during SP82G DNA entry: An initial reversible binding of one specific end is made irreversible at about 4 min. Following a latent period of 6 min attachment of the other end occurs. This second end attachment and subsequent entry of the molecule may be prevented by exposure to 4°C during the latent period. Immediately upon attachment of the second end, entry begins. The end of the molecule which attached to the bacterial cell first enters first, and the other markers follow sequentially. Entry is completed within a period of about 3 to 4 min.

During the preparation of this manuscript, the author was informed by Dr. David Dubnau of his research (in press and unpublished) concerning the pre-entry events in B. subtilis transformation. Dubnau and Cirigliano (2) observe that the pre-entry transformant complexes are initially sensitive to "Vortex-Mixer" stirring and progressively attain resistance to the treatment within the first 4 min. "Vortex mixing" in the first 4 min. removes DNA from the pre-entry complex. At about 6-7 min, the complexes achieve DNase resistance. It is likely that these times correspond respectively to the binding of the second end and the entry of DNA shown in this paper. Dubnau (personal communication) has reported a cold sensitive period in B. subtilis transformation throughout the pre-entry phase and extending beyond the period in which the DNA attains resistance to exogenous DNase. Strauss (15) has shown that cold sensitivity coincides with a period of cyanide sensitivity of the DNA-cell complex. The extended cold sensitivity of trans-
formant complexes beyond the period of establishment of DNase resistance is in marked contrast to SP82G DNA infection reported here. It suggests that perhaps transformant complexes remain dependent on a membrane association until integration, whereas, the rescue of phage DNA is independent of such an association once entry has taken place.
References


SECTION III

SOME CELLULAR ASPECTS OF DNA ATTACHMENT AND UPTAKE

Introduction

Previous work has suggested that there may exist a requirement for attachment of both ends of an infecting DNA molecule to B. subtilis cells before entry of the molecule can proceed (Section II). Four types of experiments have been undertaken which bear upon the nature of the cellular receptor sites and the process of molecular uptake.

Exposure to 47° prior to attachment of DNA (14) or within 4 min of the initial attachment (Section II) results in the loss of competence by cells, accompanied by release of attached DNA. Experiments were thus undertaken to determine a) the nature of the DNA after release, and b) whether cells which have lost competence in this manner can again become competent after brief reincubation at normal temperatures. If the loss of competence is readily reversible, it would imply that the effect is specific and may involve changes in a single, easily repaired or resynthesized, macromolecule. A more extensive disruption of the competence apparatus would be suggested by greater difficulty in reaching competence.

The possibility that two competent cells may attach to either end of an available DNA molecule and share it was suggested by Green (6). If attachment of DNA to cells is end specific and time specific, as has been reported (Section II), it would be necessary for this sort of molecule sharing to occur in a very specific manner, for after initial attachment only one distinct end of the molecule would be available for sharing. The experiments reported here attempt to define conditions under which
cells are capable or incapable of competing for DNA molecules which are attached at one end to a different cell. The genetic aspects of molecule sharing reveal which specific ends of the molecules may be shared and under what circumstances sharing occurs.

Results of the previous experiments suggest that binding of the second end of the DNA to cells can only occur after activation of a distinct binding site by the attachment of the first end of the molecule. Chloramphenicol was added to competent cultures before and after exposure of the cells to DNA in order to determine whether synthesis of new protein is involved in activation of the second binding site. No inhibitions of marker rescue by chloramphenicol would confirm the results of McAllister (13), and suggest that the process involved is the activation of a preexistent site, perhaps through conformational or electrostatic changes produced when the first end attaches.

Experiments with lysozyme and protease further delineate the location and chemical nature of binding sites. Uptake of SP82G DNA has been shown to be resistant to the effects of extracellular protease (6). The effects of lysozyme on DNA uptake are more complicated. Early experiments showed that extensive lysozyme treatment prevented DNA uptake in transformation (15). Prozorov (19) showed that light lysozyme treatment yielded a tenfold increase in the production of transformants. Later workers reported successful transformation of protoplasts under controlled conditions (18). Young's group showed that extensive lysozyme treatment prevents transformation without concomitant release of bound extracellular DNA from cells (27).

Tichy (24), Landman and associates (25, 16) reported detailed work demonstrating that, although light lysozyme treatment facilitates trans-
formation, the presence of some residual cell wall is necessary for successful penetration of the cell by infecting DNA. Lysozyme treatment of DNase insensitive DNA-cell complexes renders the DNA sensitive to DNase. Extensive lysozyme treatment does not inactivate the host cells (15), but special nutritive requirements, and perhaps also physical requirements exist for transformed protoplasts relative to untransformed protoplasts (16, 11).

The inhibitory effect of extensive lysozyme treatment may be due to sampling difficulties arising from the special requirements of transformed protoplasts (11), or the treatment may directly inhibit uptake if entry of the molecule required the presence of a finite amount of cell wall, as has been suggested (16). Physical exposure of DNA located between the cell wall and the membrane could result in loss to the media or to extracellular nucleases, or entering DNA could be trapped and stripped off with the cell wall (15). Also, removal of the cell wall could so drastically interfere with membrane structure and polarity that this alone could prevent uptake (24).

Because of the vagaries involving extensive lysozyme treatment, only light treatment (generally agreed to facilitate DNA uptake) was used in these experiments. Combined sequential treatment with lysozyme and protease might be expected to expose and destroy and protein receptor sites which would normally be protected by the cell wall structure. Regulation of the time at which protease is added permits determination of which receptor site is being affected, since binding of the two ends is time specific.

Inhibition of transformation with cyanide has been described by Strauss (23). Transformant complexes were shown to remain sensitive to
cyanide for 6 min after achieving resistance to DNase. Cyanide inhibits cellular energy producing systems, and especially those involved in membrane transport, however it is not clear whether Strauss's 6 min lag reflects a delay between achievement of DNase resistance and passage through the membrane (23), or whether passage through the membrane occurs simultaneous with achievement of DNase resistance followed by a period when the DNA remains membrane associated (and dependent upon membrane function) intracellularly. It is desirable to determine whether a similar lag period exists in the marker rescue system.
Materials and Methods

**Bacterial Strains;** Bacillus subtilis strain SB1 (17) was the host cell.

**Viral Strains;** Wild type SP82G bacteriophage (6) and four temperature sensitive double mutants of this phage, E14-H24, H167-A4, G55-H177, and H177-H201 (10) were used. The first three markers represent closely linked pairs of mutants representing the right end, middle, and left end of the genetic map respectively. The last mutant is a distantly linked pair; H177 is a left end marker and H201 is a right end marker. Double mutants are used to prevent the production of wild type infective centers due to reversion.

**DNA Isolation;** Concentrated preparations of phage were purified by centrifugation on a CsCl step gradient. DNA was extracted (4) by incubation with NaClO₄. Care was taken to prevent fragmentation by shearing. Analysis of this product by centrifugation in a 5-20% sucrose gradient (pH 7.0) showed the DNA to be uniform in size; it sedimented at a rate equal to that of whole SP82G DNA (molecular weight = 115 x 10⁶).

DNA from SP82G has been shown to be linear, nonpermuted, double stranded, and physically colinear with the genetic map (6, 7, 13).

**Competence and Media;** Competence regimes were similar to those of Spizizen (20); They have been described in detail, as well as the media used for plating and assay (6).

**Marker Rescue;** The technique of genetic marker rescue by preinfecting mutant phage has been described in detail (8). Wild type genes introduced on phage DNA are rescued by recombination with the DNA of preinfecting mutant phage, resulting in the production of a wild type infective center.
Reversibility of Initial DNA Binding: Competent cells were preinfected for 6 min with phage mutant E14-H24, or with G55-H177; multiplicity of infection (MOI) = 5 phage per cell. Wild type phage DNA was adsorbed (0.1 ug/ml for 1 min), followed by a five fold dilution in warm media. Centrifugation of cells permits assay of the supernatant for residual activity due to free unadsorbed DNA. Correction for this activity is later applied to the results.

Immediately after dilution, samples were exposed to 47° for 2 min, then centrifuged (7,000 g for 5 min) to separate the cells from the DNA released by heat treatment. The supernatant was mixed with an equal volume of fresh undiluted competent cells which had been preinfected with either E14-H24 or with G55-H177 mutant phage (MOI=5 for 6 min). The mixture was incubated (33° for 15 min) and assayed to determine the infectivity of the heat released wild type DNA. Fresh DNA of identical concentration was used in place of the supernatant as a control.

Unpreinfected competent cells were exposed to 47° for 5 min, returned to normal temperature (33°) and incubated. At 10 min intervals samples were taken, preinfected with mutant phage (6 min, MOI=5) and exposed to wild type phage DNA (1.0 ug/ml for 15 min) at 33°; then assayed for production of wild type infective centers. Fresh competent cells (not exposed to 47°) and precompetent cells just entering the final phase of the competence regime (dilution is LS media) were treated in the same manner as controls.

Competition of competent cells for genetic markers on cell attached DNA: One group of cells (A) was preinfected with phage mutant G55-H177 (6 min, MOI=5) exposed to DNA from the same mutant (G55-H177:1.0 ug/ml for 1 min) and diluted tenfold to terminate adsorption. A second group of
cells (B) was preinfected for 6 min with mutant phage E14-H24 (same conditions), exposed to DNA from the same phage mutant (E14-H24: same conditions as above) and also diluted tenfold. Samples from both groups were centrifuged at 7,000 g for 10 min, and the supernatant incubated for 10 min with an equal volume of undiluted competent cells (preinfected with phage mutant H167-A4) to assay the residual unattached DNA. Although termination of DNA adsorption by this dilution is not complete, further dilution (or use of less DNA) produces too little activity to permit detection of molecule sharing events. Results must therefore be corrected for residual DNA activity due to incomplete termination of DNA adsorption.

The first experiment involved incubating samples containing:
a) preparation A, b) preparation B, and c) mixtures of equal volumes of A and B made at 3, 6, 9 and 12 min after dilution. Samples were incubated for 15 min at 33° and then assayed for production of wild type infective centers, indicating the occurrence of molecule sharing.

In the second experiment, a volume of preparation A was incubated with an equal volume of competent cells which were preinfected with phage mutant E14-H24 and diluted tenfold but not exposed to DNA. Conversely, a volume of B was incubated with an equal volume of competent cells which were preinfected with G55-H177 mutant phage, diluted, but not exposed to DNA. After 15 min incubation at 33°, these were assayed for production of wild type centers.

In the third experiment, preparation A was sheared at 6,000 RPM for 1 min in a Sorvall Omnimixer, 5 min after attachment of DNA to the cells. This treatment releases attached DNA in active form, capable of infecting competent cells (Section II). After shearing, cells were sepa-
rated from the shear-released free DNA by centrifugation at 7,000 g for 5 min, and the pellet was resuspended in a volume of fresh media equal to the supernatant. Both the resuspended cells and the supernatant containing free DNA were exposed to an equal volume of preparation B for 15 min at 33° and assayed for the production of wild type infective centers. Preparation B was sheared and the analogous converse experiment performed.

A final competition experiment involved preinfecting competent cells with phage mutant H177-H201 (6 min, MOI=5). Cells were then exposed to wild type phage DNA (10 ug/ml for 1 min), and adsorption was terminated by a hundredfold dilution. A 10 ml portion was centrifuged at 7,000 g for 10 min and the supernate was incubated with 10 ml of undiluted competent cells preinfected with phage mutant H167-A4 to determine the residual activity of unattached DNA.

Six min after dilution, samples from the incubating H177-H201 infected cells were added to equal volumes of; a) undiluted competent cells which were not preinfected, b) undiluted competent cells preinfected with phage mutant E14-H24 (6 min, MOI=5), or c) media containing no cells or DNA, (as control). Samples were then reincubated for 10 min to permit sharing of the DNA attached to the H177-H201 infected cells with the other cells, and then the mixtures were assayed for the production of wild type infective centers.

**Sensitivity of competent cells to lysozyme and protease:** Competent cells were exposed to a light dose (50 ug/ml) of egg white lysozyme (Grade I, recrystallized, Sigma) for 20 min. Five min before the end of this period, cells were preinfected (MOI=5) with mutant phage H167-A4, and 6 min later exposed to wild type phage DNA (10 ug/ml). Samples were treated with protease (500 ug/ml *Streptomyces*, repurified, Sigma) at 2
min intervals, beginning 2 min before addition of the DNA and continuing until 12 min after DNA addition. At 16 min, all samples were assayed for production of wild type infective centers. Lysozyme and protease treatments under these conditions were shown to have no adverse effect on the viability of the preinfected cells. Control cells consisted of: a) omission of the lysozyme treatment, b) omission of the protease treatment, and c) omission of both treatments.
Results and Discussion

Within the first 4 min after attachment of DNA to the cell, exposure to 47° results in release of all bound DNA (Section II). Exposure to 50° has also been shown to cause loss of competence in cells prior to DNA exposure (14). The results of the present experiment demonstrate that the released DNA retains activity equivalent to whole fresh DNA, and that both ends of the molecule are recovered with equal frequency after heat release (Table III). This suggests that DNA is released as intact whole molecules, essentially identical to those which have never been attached to cells.

Competence reappears in heat treated cultures at a rate comparable to the initial appearance of competence in precompetent cultures (Fig. 5). This suggests that previously competent cells may be permanently inactivated, that reappearance of competence in the culture reflects attainment of competence by previously non-competent segments of the population. These data are also consistent with the idea that heat treatment shocks competent cells into a state equivalent to early pre-competence, and that competence may be reattained by the same cell upon reincubation.

Short periods of reincubation do not result in recovery of competence, as would be expected if the effect were readily reversible (such as the denaturation of a specific protein which could be readily resynthesized). More likely the physiological effects of the treatment are fairly extensive. If protein denaturation is involved, as it may well be, there is also concommittent loss of capacity to resynthesize the specific protein involved. Alternatively, the effect may arise from less specific changes; electrokinetic potential of the cell surface, membrane
TABLE III. Infectivity of DNA released from cell attachment by 47° exposure

Yield of wild type infective centers from cells preinfected with mutant markers

<table>
<thead>
<tr>
<th></th>
<th>G55-H177</th>
<th>H167-A4</th>
<th>E14-H24</th>
</tr>
</thead>
<tbody>
<tr>
<td>heat released DNA</td>
<td>614.5</td>
<td>716.5</td>
<td>730.0</td>
</tr>
<tr>
<td>control DNA (free)</td>
<td>633.0</td>
<td>721.5</td>
<td>724.0</td>
</tr>
<tr>
<td>control supernate</td>
<td>28.0</td>
<td>35.0</td>
<td>9.5</td>
</tr>
<tr>
<td>(no 47° treatment)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5. Recovery of competence by cell culture exposed for 5 min to 47°. Competent cells were exposed to 47° for 5 min, then reincubated at normal temperature (33°). Samples taken at 20 min. intervals show recovery of ability to perform marker rescue.

○ cells previously competent then exposed to 47°.
□ competent cells not treated with 47°.
△ precompetent cells entering final phase of competence regime (diluted in LS; 90 min before achieving competence).
polarity, or membrane structure.

The competition studies were undertaken to determine conditions under which cells would or would not be able to share DNA molecules by competing for attachment of the free second end. Cells preinfected with mutant phage and then exposed to DNA from the same phage mutant are capable of absorbing the DNA but cannot produce wild type infective centers. If two cell populations, infected with mutants on opposite ends of the genetic map, are able to share molecules, production of wild type infective centers would be possible. The results of the first experiment (Fig. 6) indicate that this is the case. Cells (infected with phage and DNA mutant at one end of the genetic map) are capable of competing for wild type alleles on the free end of DNA attached to other cells (infected with phage and DNA mutant at the other end of the genetic map).

The loss of ability to compete after 9 min suggests that after this time there are no free DNA ends available for cells to compete for. This confirms earlier results that both ends of the molecule are attached to cells by 10 min (Section II).

In this experiment it is not possible to determine which genetic markers are being shared, or whether both ends of the molecule are capable of sharing. To determine which markers are involved, cells infected with phage and DNA mutant at one end of the molecule were mixed with cells infected with phage mutant at the other end, but not exposed to DNA. The results (Fig. 7) show that no wild type infective centers were produced by either end of the molecule. This does not show which markers are shared in the previous experiment, but it does suggest that the second end of the molecule can only bind the cells which already have DNA attached to them. A reasonable explanation for this would be that activa-
Figure 6. **Sharing of DNA molecules by cells infected with different mutant phage and phage DNA.** Cells infected with phage and DNA mutant at one end of the DNA molecule were mixed with cells infected with phage and DNA mutant at the other end of the molecule. Production of wild type infective centers demonstrates sharing of wild type markers between the two cell populations.

- □ cells infected with phage and DNA mutant E14-H24.
- ■ cells infected with phage and DNA mutant G55-H177.
- Δ mixture of equal volumes of both above cell populations.
WILD TYPE YIELD PER ML

MINUTES AFTER MIXING
Figure 7. Inability of cells without attached DNA to compete for or share DNA attached to other cells. Lack of significant production of wild type infective centers reflects inability of phage preinfected cells with no previous DNA exposure to make use of available wild type markers on DNA attached to other cells.

- Cells infected with phage and DNA mutant E14-H24.
- Cells infected with phage and DNA mutant G55-H177.
- Cells infected with phage and DNA mutant E14+H24 mixed with equal volume cells infected with phage only, mutant G55-H177.
- Cells infected with phage and DNA mutant G55-H177 mixed with equal volume cells infected with phage only, mutant E14-H24.
WILD TYPE YIELD PER ML

MINUTES AFTER MIXING
tion of the receptor site for the second end depends upon previous binding of DNA at the initial attachment site. This will be pursued after the next experiment.

Physical shearing has been shown to remove most, but not all, attached DNA from the cell surface. (Section II). If the previous hypothesis about second end binding is correct, and if the piece of DNA which remains cell attached after shearing is sufficient to activate the second site, sheared cell-DNA complexes may be used to determine which markers are being shared. Cells were infected with DNA and phage mutant at one end of the molecule. The attached DNA was removed by shearing and centrifugation. After centrifugation, both the sheared cells (the c fraction) and the supernatant containing the free DNA (the s fraction) were exposed in turn to cells infected with DNA and phage mutant at the other end of the molecule. If initial attachment of the DNA is end specific it should now be possible to determine which end binds initially (and so cannot be shared) and which end binds last (and can be shared).

In the s fraction mixture, complementation to produce a wild type infective center must involve the gene which is mutant in the competing cells (i.e. the gene which is not mutant in the sheared cells), for the competing cells are the only cells present in the mixture, and hence the only possible source of wild type infective centers. In the c fraction mixture the situation is reversed for there are two types of available cells but only one available DNA; that from the competing cells. Production of a wild type infective center in this case must involve the gene which is not mutant in the DNA (i.e. which is not mutant in the competing cells but is mutant in the sheared cells) for these are the only wild type alleles available in the mixture. If there is no specificity
TABLE IV. Molecule sharing between DNA infected cells and cellular (c) and supernate (s) fractions of sheared DNA infected cells

<table>
<thead>
<tr>
<th>Yield of wild type infective centers/0.1 ml mixture</th>
<th>a) sheared preparation A</th>
<th>b) sheared preparation preparation B</th>
<th>B; preparation A</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole fraction alone</td>
<td>0.0</td>
<td>21.0</td>
<td>whole</td>
</tr>
<tr>
<td>s fraction alone</td>
<td>7.5</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>unsheared (whole) component alone</td>
<td>17.5</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>c fraction mixture</td>
<td>351.0</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td>s fraction mixture</td>
<td>13.5</td>
<td>243.5</td>
<td></td>
</tr>
</tbody>
</table>

1 When A is sheared, the B component is whole, and vice versa.
2 Mixed sc fraction of the sheared prep. with other (whole) preparation
3 Mixed s fraction of the sheared prep. with other (whole) preparation
of end attachment, complementation will produce wild type infective centers in both c and s fraction mixtures for all types of cells and DNA will be available in all mixtures. The results for these experiments (Table IV) show conclusively that there exists a specificity to the attachment of ends and that the marker involved in molecule sharing in both instances is the marker G55-H177. Previous experiments also concluded that this end of the molecule binds to the cell last (Section II).

A final competition study was designed to confirm that competent cells with no previous DNA exposure are incapable of competing for attachment of the second end of the molecule. The phage mutant H177-H201 is mutant at both ends of the molecule. If DNA entry proceeds linearly, as has been amply demonstrated (5, 21, 22), cells preinfected with this phage would require uptake of the entire DNA molecule to permit production of a wild type infective Center. Sharing either end of the molecule would decrease the yield of wild type infective centers unless competing cells are capable of using the markers they bind to produce their own wild type infective centers. This is prevented by using nonpreinfected competent cells, since they require uptake of several complete phage genomes to produce a wild type infection. Cells preinfected with E14-H24 are also effective, since the E14-H24 end of the molecule is the end of initial attachment and is therefore irreversibly bound to the first cells and unavailable to competing cells under the conditions used.

The results of this experiment (Table V) show that competition does not occur in either experimental condition with cells which have not been previously exposed to DNA, confirming the previous results.

Exposure to chloramphenicol at times immediately before or after initial attachment of the DNA molecule, does not inhibit successful
TABLE V. Production of wild type infective centers by cells preinfected with phage mutant H177-H201 and wild type DNA, then exposed to competing cells at 6 and 10 min after DNA exposure

Recovery of wild type infective centers per 0.1 ml after competition with:

<table>
<thead>
<tr>
<th>min.</th>
<th>non-preinfected cells</th>
<th>E14-H24 phage infected cells</th>
<th>no competing cells present</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>264</td>
<td>369</td>
<td>287</td>
</tr>
<tr>
<td>10</td>
<td>277</td>
<td>328</td>
<td>300</td>
</tr>
</tbody>
</table>
TABLE VI. Effect on marker rescue of chloramphenicol exposure (100 ug/ml) at various times before and after DNA addition

Production of wild type infective enter per 0.1 ml;

<table>
<thead>
<tr>
<th>min.</th>
<th>Chloramphenicol present</th>
<th>Chloramphenicol absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6</td>
<td>672</td>
<td>693</td>
</tr>
<tr>
<td>-3</td>
<td>614</td>
<td>689</td>
</tr>
<tr>
<td>0</td>
<td>633</td>
<td>649</td>
</tr>
<tr>
<td>+3</td>
<td>659</td>
<td>602</td>
</tr>
<tr>
<td>+6</td>
<td>597</td>
<td>654</td>
</tr>
<tr>
<td>+9</td>
<td>683</td>
<td>667</td>
</tr>
<tr>
<td>+12</td>
<td>642</td>
<td>618</td>
</tr>
</tbody>
</table>
molecular uptake in competent cells (Table VI). Protein synthesis is therefore not required for activation of the second binding site, since McAllister (13) has shown that chloramphenicol in the concentrations used inhibits cellular function. Two other possibilities have already been mentioned; a) binding at the second site may not involve a protein, or b) binding may involve a protein which is already present in active form, and is activated by conformational or electrostatic events involving binding of the initial end. The latter situation would be particularly appropriate if both attachments occurred at independent sites on a single protein molecule.

The unfavorable electrokinetic potential of the exterior cell surface (9) and the facilitative effects of light lysozyme treatment on transformation (19) suggest that DNA may attach through gaps in the cell wall (27) rather than on the external cell wall surface. The finding that shearing of bound DNA leaves a small residual portion of the molecule attached (Section II) may reflect protection of DNA near the attachment site by the surrounding cell wall. Transfection is not inhibited by protease (6) which suggests either that binding sites are not protein in nature or they are protected from enzyme action. Protection from protease would be easily accomplished if receptors were located between cell wall and membrane.

To determine whether a protected protein receptor may be present, experiments with lysozyme and protease were undertaken. After light treatment of recipient cells with lysozyme, extensive protease treatment is observed to inhibit marker rescue if applied before attachment of the DNA molecule (Fig. 8). After DNA adsorption, protease treatment is no longer effective. Several explanations of this result are
Figure 8. **Early sensitivity of DNA receptor site to combined treatment with protease and lysozyme.** Competent cells were exposed lightly to lysozyme for 20 min before exposure to DNA. At various times before and after DNA exposure, cells were heavily treated with protease. Lack of ability to perform marker rescue when light lysozyme treatment is combined with protease treatment before DNA attachment suggests the existence of a protein receptor site normally protected by the cell wall.

- ○ competent cells treated only with lysozyme
- □ competent cells treated only with protease
- ▲ competent cells treated with neither lysozyme or protease
- ● competent cells treated with both lysozyme and protease
possible. Protein receptors may be protected by steric hindrance due to the attached DNA molecule. There is no indication of any protease sensitivity for the second binding site. It is not clear whether this implies that the second site is not protein, or if it becomes protected simultaneously with the first site by virtue of physical proximity. Another suggestion is that neither of the irreversible binding events involve proteins, but that the initial attachment does. After an initial (heat reversible) electrostatic attachment (protein mediated), both ends may bind directly to the membrane itself, facilitating entry.

Alternatively, all attachments could occur on a single protein molecule. The pneumococcal CF protein has been shown to have a net positive charge (12). It appears to act as a catalyst in transformation, binding first to cells and then attaching to DNA molecules (26). While it would be tempting to suggest an analogous (but protected) factor of this sort in B. subtilis, information to date does not warrant it. Although competence specific and competence inducing proteins have been isolated from B. subtilis (1, 2) the nature of their role in the process is not known.

Finally, experiments with cyanide indicate inhibition of marker rescue at early time (Fig. 9). The times at which individual markers become resistant to cyanide inhibition correspond closely to times at which the same markers become insensitive to DNase (Section II). DNase resistance probably reflects passage of the DNA molecule from the extracellular environment into protected space inside the cell wall (3). Cyanide resistance is most likely conferred when transport through the membrane occurs and membrane association ends (23). The close correspondence of these events in time raises the possibility that both cell wall and membrane are traversed simultaneously, in a single event, in
Figure 9. **Achievement by cell-DNA complexes of resistance to cyanide treatment for specific marker pairs.** Cyanide apparently interferes with membrane transport and prevents entry of the DNA molecule through the cell membrane. Resistance to cyanide (0.025M) by genetic markers is achieved in the order in which they have previously been suggested to enter the cell and at the same time that they have also been shown to achieve resistance to DNase (Fig. 3H).

- ● control cells not exposed to cyanide (all markers averaged)
- □ cells infected with phage mutant E14-H24 and wild type DNA
- △ cells infected with phage mutant H167-A4 and wild type DNA
- ○ cells infected with phage mutant G55-H177 and wild type DNA
marker rescue.

Strauss has observed a 6 min lag between achievement of DNase resistance and cyanide resistance in transformation (23). There are two plausible explanations for this discrepancy. Transforming DNA may be held for some time in an intermediate state, having traversed the cell wall but remaining outside the membrane. Erickson (3) has suggested that, since competent cells are in a state of biosynthetic latency (not actively synthesizing DNA), transforming DNA may be held in a DNase resistant state outside the cell membrane until synthesis resumes. If transport of DNA across the cell membrane is dependent upon the presence of actively synthesizing DNA in the cell, this explains why there is no lag in marker rescue. Synthesis of DNA by the preinfecting phage is active at the time when DNA attaches and enters the cell (Green, personal communication).

The other possibility is that the cyanide sensitive period in transformation includes more than just entry of the molecule through the membrane. Membrane transport may occur with no lag, as it does in marker rescue, but there may be a period of continued membrane association in transformation required for recombination which is cyanide sensitive. The absence of this continuing membrane dependency in marker rescue could be attributed to a number of inherent differences between the two systems.

In conclusion, what these experiments do show is that DNA released by 47° exposure is active and the whole molecule is released. Cultures which have lost competence by 47° exposure apparently revert to stages of early precompetence. Experiments confirm that both ends of infecting DNA molecules attach to the cells, and that the attachment is
end specific and time specific. Attachment of DNA at the site of initial binding is required to activate the site responsible for the second end binding. The two sites are distinct. Activation of the second site does not involve new protein synthesis. The presence of a protein or proteins, normally protected by the cell wall, is required for successful DNA infection. After initial binding of the DNA, binding sites are no longer sensitive to protease following light lysozyme treatment of the cells. This may indicate protection due to steric hindrance from the bound DNA, or may indicate that later binding events are not protein mediated. Entry of the DNA molecule is energy dependent. Traverse of the cell wall and membrane transport appear to occur as a single act.
References


SECTION IV

PRELIMINARY EXPERIMENTS ON THE ROLE OF THE INFECTING DNA MOLECULE BEFORE ENTRY

Introduction

In comparison with the cellular aspects of DNA uptake, the role of the extracellular infecting DNA molecule appears quite simple. DNA has traditionally been assigned a passive role in the attachment and entry process. If Strauss's interpretation of the cyanide sensitivity of DNA entry (12) is correct, cellular transport mechanisms are actively involved in transporting the molecule into the cell. Uptake has been shown to involve DNA denaturation in pneumococcus (4, 8) and some degree of denaturation has been shown to be associated with uptake in Bacillus subtilis as well (1, 2). In the attachment process, the role of the DNA consists mainly of being acted upon by cellular products, competence factors, attachment sites (6).

A second reason why the role of the infecting DNA molecule has been generally overlooked is that it is much more difficult to observe, manipulate and analyze the activities of free molecules than it is to deal with cells. It is a comparatively simple matter to isolate proteins, destroy receptor sites, and inhibit specific physiological functions (though many will no doubt take exception). To observe specific structural and conformational aspects of molecular interactions, and to manipulate them effectively for experimentation, is more difficult.

The attachment specificity of ends of the DNA molecule (Section II), as well as the evidence for denaturation of the molecule previously mentioned, invite further experimentation. The preliminary experiments
reported here raise more questions than they answer, however, they do offer some insight into these two aspects of the uptake process. In the first experiment, the uptake of genetic markers on DNA which has been fragmented by physical shearing is studied. The second experiment deals with the effect of antiserum directed against single stranded DNA on the process of molecular uptake.

Physical and genetic characterization of sheared infective SP82G bacteriophage DNA has been reported by Green (6). Under controlled shearing conditions, the DNA is selectively fragmented into pieces with a molecular weight just half that of the native whole molecule. The halves are infective, individual markers and close doubles retain above 75% of the native marker contributing ability in marker rescue. Genetic analysis places the mean breakage point just to the right of the marker H167 (see Fig. 1). Further controlled shearing produces material with molecular weight of one fourth that of the original whole molecule. These pieces also retain some marker contributing ability in marker rescue, and genetic analysis has identified specific regions of breakage for the second shear event. The present experiments make use of these specifically fragmented molecules to observe the entry of genetic markers under conditions similar to those used earlier to observe entry of whole bacteriophage DNA (SectionII).

Inhibition of transformation has been accomplished using antiserum directed against single stranded DNA (2). The inhibition reaction has been suggested to occur intracellularly, because prolonged incubation of the antiserum with recipient cells is required prior to DNA exposure. With less opportunity for interaction between cells and antibody, inhibition is not effective. It would seem reasonable that this
effect might result from adsorption of antibody molecules to the cell surface, however Erickson feels it more likely that the antibody is actually absorbed into the host cell. He has shown that competent cells are capable of binding isotopically labelled rabbit gamma globulin, as well as a number of other macromolecules (2). On the surface of the matter, it would appear that other explanations are possible if not preferable. In the experiments which are reported here, conditions which are decidedly extracellular prevail. Under these conditions it can be shown that, beyond not inhibiting DNA attachment and uptake, as Erickson has suggested, the antiserum actually facilitates DNA uptake in the marker rescue process.
Materials and Methods

**Bacterial Strains:** B. subtilis strain SB1 (10) was the host cell.

**Viral Strains:** Wild type SP82G bacteriophage (5) and three temperature sensitive double mutants of this phage, E14-H24, H167-A4, and G55-H177 (8) were used. These mutants represent closely linked pairs of mutants representing the right end, middle, and left end of the genetic map respectively. Double mutants are used to prevent the production of wild type infective centers due to reversion.

**DNA Isolation:** Concentrated preparations of phage were purified by centrifugation on a CsCl step gradient. DNA was extracted (3) by incubation with NaClO₄. Care was taken to prevent fragmentation by shearing. Analysis of this product by centrifugation in a 5-20% sucrose gradient (pH 7.0) showed the product to be uniform in size. It sedimented at a rate equivalent to that of whole SP82G DNA (molecular weight = $6 \times 115 \times 10^6$).

**Competence and Media:** Competence regimes were similar to those of Spizizen (11); they have been described in detail, as well as the media used for plating and assay (5).

**Marker Rescue:** The technique of genetic marker rescue by preinfecting mutant phage has been described in detail (7). Wild type genes introduced on phage DNA are rescued by recombination with mutant DNA from preinfecting bacteriophage. Rescue of wild type markers is detected by production of a wild type infective center.

**Shearing of DNA and Analysis of Shear Products:** Following the method of Green (6), 3 ml samples of DNA at a concentration of 16.2 ug/ml (in 0.15 M NaCl + 0.015 M sodium citrate + 0.01 M phosphate buffer, pH 7.0).
were stirred in a Virtis microhomogenizing flask with a single Virtis micro stainless steel blade attached to a Heller GT21 stirring motor. The rate of stirring was determined by continuous monitoring of the rotor shaft with a strobometer (Strobotac). All samples were stirred at 4°C for 20 min, at rotor speeds of 2300 or 4600 RPM.

Shear products were sedimented in a 5-20% sucrose gradient (pH 7.0) at 40,000 RPM for 105' in a SW65 rotor. Sedimentation coefficients were determined relative to native whole SP82G DNA. Genetic analysis of the sheared DNA was also undertaken. The infectivity of the three close double mutants, E14-H24, H167-A4, and G55-H177 was determined relative to whole native DNA. Infectivity of the distantly linked markers H177-H167 and H167-H24 was examined relative to whole DNA to determine the extent of dislinkage between the various regions of the genome. Genetic analyses were conducted as described by Green (6) competent cells were preinfected with the appropriate mutant phage (MOI=5 for 6 min), then exposed to phage DNA (0.07 ug/ml) for 10 min. They were then assayed at 47°C for the production of wild type infective centers.

Uptake of Genetic Markers on Sheared DNA; Experiments similar to those previously reported for whole DNA (Section II) were undertaken using the sheared DNA. Competent cells were preinfected with mutant phage (MOI=5 for 6 min) and then exposed to the sheared phage DNA (1.0 ug/ml for 1 min). Adsorption was terminated by a hundredfold dilution in warm media. At 2 min intervals, 10 ml were removed for experimentation. Samples were exposed to four sets of conditions; (a) Samples in 1 M MgCl₂ were incubated with 1 mg/ml of DNase I for 1 min at 37°. (b) Samples were sheared in a Sorvall Omni-Mixer for 30 sec at 11,500. (c)
Samples were chilled to 4° and held at this temperature for 10 min. (d) Control samples were plated immediately at 47°. Immediately after treatment, all samples were incubated at 47° to assay the yield of wild type infective centers.

Preparation of Antiserum: Young 4 lb rabbits were injected with antigen composed of equal parts of whole SP826 DNA (300-350 ug/ml, denatured by heating to 100° for 10 min and chilling rapidly then complexed with methylated Bovine serum albumin) and Freund's Complete Adjuvant (Pentex, Inc.). The antigen mix was stirred to form an emulsion and interdermal injections were made on the hind foot pads, plus intradermal injections in the back. Intramuscular injections were made into the hind leg muscles. A total of about 500 ug of DNA antigen was injected per rabbit by this treatment. The treatment was repeated twice at weekly intervals. One week after the third injection, 1.5 ml of denatured whole SP82G DNA (300-350 ug/ml) was administered intravenously. The following week, animals were test bled, and 25 ml of blood was collected from each animal the following day. After rimming and clotting, the serum was isolated by centrifugation. It was held at 60° for 40 min to destroy complement and any heat labile enzymes which were present, and it was assayed for antibody activity against single and double stranded DNA.

Double diffusion plates were used for preliminary determination of antibody titre and specificity. A twofold dilution of the serum complexed with an equal volume of denatured SP82G DNA (32 ug/ml). No visible reaction was observed between native (undenatured) double stranded phage DNA and the antiserum. Further determination of the binding characteristics of the antibody was undertaken by Green (personal
communication) in the course of other experiments. He studied the ability of the antibody to complex with and precipitate radioactive \(^{32}P\) labelled DNA. Saturating concentrations of antiserum were shown to precipitate over 50% of isotope label present as single stranded (denatured) DNA, and about 10% of isotope label present as double stranded (native) DNA. Overnight incubation of DNA-antiserum complexes at 0° resulted in the release of DNA from the complex, observed as loss of ability to precipitate \(^{32}P\) labelled DNA.

**Effect of Antiserum on Marker Rescue:** Cells were preinfected with mutant phage (MOI=5 for 6 min) and exposed to DNA (1.0 ug/ml) for 1 min. Adsorption was terminated by a tenfold dilution in warm media. Antiserum to complex with 10.0 ug/ml denatured DNA was added to samples at times immediately before and after exposure of cells to DNA. Control samples were not exposed to antiserum. Samples were incubated for 15 min after the dilution (at 33°), and then assayed for the production of wild type infective centers.

To determine that the observed effects were due to specific antibody action, antiserum was swamped with excess denatured DNA and the complex was precipitated by centrifugation. This process was repeated twice to insure that all active complexing antibody was inactivated and removed. The effects of the thus inactivated antiserum on marker rescue were studied in the experiment above. To insure that the observed effects were not due to general effects of proteins in the antiserum, 0.2 ml of 1.0% bovine serum albumin was added to 1 ml samples in place of 0.2 ml antiserum in the above experiment and the effect on marker rescue was observed.

An experiment was conducted to determine the specific nature of
the antibody activity on the DNA uptake process. Wild type DNA (native) was exposed to a tenfold excess of antiserum and the complex precipitated by centrifugation with albumin. The process was twice repeated on the remaining supernate. The DNA remaining in the supernate was then used to perform marker rescue, as in the previous experiments. The production of wild type infective centers by this DNA was compared to results obtained under identical circumstances using DNA which had not been previously treated with antibody.

In the previous experiment, no antiserum was added during the course of the DNA attachment and uptake, as had been done in the earlier experiments. The only treatment with antibody preceeded the exposure of DNA to cells and it involved only the isolated DNA. A final experiment was done, also using antibody pretreated and antibody unpretreated DNA to perform marker rescue. However, this time, both samples also received treatment with a tenfold excess of antibody at various times immediately before and after attachment of the DNA molecule. These experiments theoretically complement Erickson's research (2) which deals with the effects on transformation of preincubating competent cells with the antiserum.
Results and Discussion

Physical characterization of the sheared SP82G DNA by centrifugation yields results similar to those reported by Green (6). Shearing under the described conditions at 2300 RPM produces a quantitative shift of the bulk of the DNA to a position corresponding to half the native molecular weight. Shearing at 4600 RPM results in a quantitative shift to a position corresponding to a fourth of the original molecular weight. A single DNA peak is observed after each shear event; \( S_{20w} \) values calculated relative to the known \( S_{20w} \) of native whole material (=63.2 (6)) are 49.2 and 30.5 respectively, compared to values of 47.0 and 33.2 observed by Green (6). Results of the genetic characterization (Table VII) are also compatible with those of Green. 49.2s DNA retains about 75% activity for close marker pairs, and about 70% activity for the pair H177-H167, indicating that little breakage has occurred between these markers. H167-H24 retains only about 35% activity, indicating that a significant amount of dislinkage has occurred between these markers, confirming Green's finding that the specific shear point is to the right of the marker H167 (6).

Because of the heterogeneity of the DNA population, it is necessary to demonstrate that experiments are being performed under conditions in which a single molecule infects a single cell. This is demonstrated by the linearity of a DNA concentration response curve under the conditions of adsorption used in the experiment (Fig. 10).

The 47°, 4°, shear, and DNase sensitivity results for the individual marker pairs on 49.2s DNA (Fig. 11) may be compared to similar results reported previously for markers on whole DNA (Fig. 3). End markers
### TABLE VII. Loss of wild type marker contributing ability following shearing of DNA.

Percent rescuable wild type activity relative to unsheared DNA

<table>
<thead>
<tr>
<th>Marker pair</th>
<th>First shear (2300 RPM)</th>
<th>Second shear (4600 RPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G55-H177</td>
<td>85%</td>
<td>45%</td>
</tr>
<tr>
<td>H167-A4</td>
<td>79%</td>
<td>42%</td>
</tr>
<tr>
<td>E14-H24</td>
<td>74%</td>
<td>29%</td>
</tr>
<tr>
<td>H177-H167</td>
<td>71%</td>
<td>12%</td>
</tr>
<tr>
<td>H167-H24</td>
<td>35%</td>
<td>15%</td>
</tr>
</tbody>
</table>
Figure 10. Concentration response curve for marker rescue using DNA sheared to 49.2s. DNA of various concentrations was used to perform marker rescue under the conditions described in the text, and the production of wild type infective centers was assayed. Linear increase in the production of wild type centers with increasing DNA concentration indicates that each additional increment of DNA produces a proportional increase of wild type centers, thus additional molecules infect new cells rather than reinfecting cells already infected with DNA. This assures that one cell is being infected with a single DNA molecule, statistically speaking, in these experiments.
(E14-H24 and G55-H177) behave in identical fashion on whole and halved DNA. The behavior of the middle marker (H167-A4) approximates the behavior of the right end marker (E14-H24) much more closely with halved DNA than is observed using whole DNA. This result was anticipated, since shearing to the immediate right of the marker H167-A4 effectively transforms it from a middle marker to a marker representing the right end of the left half. In achievement of DNase resistance, this marker behaves more like a right end marker than E14-H24; entering before it. This also might be expected if H167-A4 is closer to the right end of the left half than E14-H24 is to the right end of the right half, which may occur.

The time representing the lag period between attachment of the first and the second end of the DNA, (the time difference between achievement of resistance to 47° and 4° for all markers) is unchanged by shearing molecules in half, suggesting that the length of the infecting DNA molecule is not the determining variable for this reaction. Achievement of DNase resistance (entry) does not take quite as long with half molecules; it is complete by 15 min.

No information about the origin of the attachment specificity for individual ends was obtained. The sheared ends apparently behave quite effectively as left or right ends, depending upon which half of the molecule they end up in. There is perhaps a graded attachment specificity for certain nucleotide sequences, the natural left and right ends representing opposite poles of the gradient. Sheared ends, no special affinity for either site, might then be expected to behave as intermediates.

Experiments with quartered DNA (not reported) was inconclusive,
Figure 11. Attachment and entry of sheared SP82G phage DNA.

Competent cells were preinfected with mutant phage and exposed to sheared wild type phage DNA as described in the text. Samples were taken at various times and exposed to conditions previously described in similar experiments using whole DNA (Fig. 3, section 1), then assayed for the production of wild type infective centers.

A) samples plated directly at 47° at experimental times
B) samples chilled to 4° for 10 min, then plated at 47°
C) samples sheared at 11,500 RPM for 30 sec, then plated
D) samples exposed to DNase I (1 mg/ml for 1 min) and plated

○ cells preinfected with marker E14-H24
△ cells preinfected with marker H167-A4
□ cells preinfected with marker G55-H177
Figure 12. **Effect of antiserum directed against single stranded SP82G phage DNA on marker rescue.** Cells preinfected with mutant phage markers and exposed to wild type phage DNA were put in the presence of a tenfold excess of antibody (enough antibody to complex 10 times the amount of DNA present if all were single stranded) at various times after addition of DNA to the cells. Yield of wild type infective centers is presented as percent of the yield when no antibody is added.

- △ cells preinfected with mutant phage E14-H24.
- ○ cells preinfected with mutant phage H167-A4.
- □ cells preinfected with mutant phage G55-H177.
- ■ Bovine serum albumin used in place of antiserum; results of all markers averaged.
- ● Inactivated antiserum, as described in text; results of all markers averaged.
PERCENT WILD TYPE YIELD RELATIVE TO UNTREATED SAMPLES

MINUTES

12  6  3  0  3  6  9  12

0  200  300  400  500
owing probably to the lower activity of individual markers and the
greater heterogeneity of the molecular population of fragments.

Antibody directed against single stranded DNA (Fig. 12) en­
hances DNA attachment and uptake under the conditions used in these
experiments. Late entering markers are facilitated more than earlier
entering markers. Controls (Fig. 12) demonstrate that this effect is
not due to general effects of proteins, or to nonspecific components
of the antiserum.

The physical and genetic effects of treating native whole SP82G
phage DNA with excess antibody (Fig. 13), confirm Green's results
(personal communication) that there exists a residual antibody activity
which is not specific to only single stranded DNA. Some enhancement
of marker rescue is observed if the infecting DNA is pretreated with
antibody, however this enhancement is not marker specific. Addi­
tion of antibody during DNA attachment and uptake, even when antibody
pretreated DNA is used to infect, does produce the marker specific
pattern of enhancement (Fig. 14). Indeed, if the infecting DNA has
been antibody pretreated, the marker specific enhancement is even
greater than that observed when DNA has had no previous antibody ex­
posure.

These results suggest that the facilitation of marker rescue
by antibody is a two phase process, involving both marker specific and
nonspecific effects. The nonspecific effect observed in the case of
DNA pretreatment may result from the removal of inactive DNA fragments
which compete with active fragments for attachment sites. DNA with
overlapped (single stranded) ends is uninfective. But if it were capa­
ble of binding to attachment sites, specific removal of these molecules
Figure 13. Effect of pretreating infecting DNA with antiserum directed against single stranded phage DNA. Infectivity of DNA after successive precipitations with antiserum directed against single stranded DNA was studied. Each antiserum treatment removed DNA from the preparation, as observed by the successive reduction in O.D. 260. Activity of treated DNA, compared to the activity of untreated DNA at a concentration equal to the initial concentration of the DNA also declines slightly, indicating removal of some active double stranded material by the precipitation. Activity of the treated DNA compared to the activity of untreated DNA of concentration equal to the final concentration of treated DNA after each precipitation increases slightly, indicating a possible secondary enhancement mechanism in addition to removal of inactive competing molecules.

- optical density (260) of treated DNA preparation relative to optical density of DNA prior to any treatment.

- activity of treated DNA preparation (ability to perform marker rescue) relative to activity of initial DNA preparation prior to any treatment.

- activity of treated DNA preparation relative to activity of untreated DNA at concentration equal to the final concentration of treated DNA remaining after each individual precipitation.
Figure 14. **Marker specific enhancement of marker rescue due to antiserum treatment of DNA-cell complexes using antiserum pretreated DNA.**

Timed experiments such as those reported in Figure 12 are repeated using DNA which has been pretreated with the anti-single stranded DNA antiserum as described in the text. Comparison of these results with those reported in Figure 12 show that enhancement of marker rescue is greater when antiserum treated DNA is used. The increase is approximately what would be expected from the data given in Fig. 13.

- △ cells infected with mutant phage E14-H24.
- ○ cells infected with mutant phage H167-A4.
- □ cells infected with mutant phage G55-H177.
PERCENT WILD TYPE YIELD RELATIVE TO UNTREATED SAMPLES
by the antibody could produce a general enhancement of DNA infection such as is observed. Another possibility is that the presence of residual serum in the antibody treated DNA is responsible for the effects. This may involve only a single specific component of the serum. Finally partial or temporary binding of active DNA and antibody may produce structural changes in the DNA which facilitate uptake of the antibody modified DNA.

The protection effect which shows marker specificity is even more interesting theoretically, since the enhancement may be related to the actual uptake process. A simple model might suggest protection of attached or entering DNA by the antiserum. Protection would thus be greatest for those markers which entered last, since they would remain under the protecting influence of the antibody for the longest time. It is not necessary to point out that there exist several extracellular nucleases which could act on attached extracellular DNA in the absence of some protection of this sort (13). One criticism of this reasoning is that it postulates the existence of an activity gradient for markers along the DNA molecule under ordinary conditions. Such a gradient does not seem to exist.

Two mechanisms for this sort of protection can be envisioned. The antibody may bind weakly to the double stranded attached DNA along its entire length, being displaced at the point of entry as the molecule is pulled into the cell. It may also bind to a specific denatured (single stranded) segment (3) of the molecule. Such a segment would be especially sensitive to nuclease attack. If DNA is denatured during entry, and especially if denaturation slightly precedes entry, this sort of protection would seem reasonable. If antibody-DNA binding were
not too tight, the protein might be progressively displaced during entry as the denaturation progressed along the molecule at the same time. As with the other model; the observed results (with late entering markers protected most) would be expected from this sort of protection.
References

1. Dubnau, D. and C. Cirigliano (1972). Fate of transforming DNA following uptake by competent *Bacillus subtilis* III. Formation and properties of products isolated from transformed cells which are derived entirely from donor DNA. J. Mol. Biol. 64 (in press).


BIOGRAPHICAL DATA

Name in Full: Gordon Lee Williams

Date of Birth: September 9, 1947

Place of Birth: Mechanicsburg, Pennsylvania

Secondary Education: Mechanicsburg Area High School

Collegiate Institutions Attended Dates Degrees
Lehigh University 9/65 - 6/69 B.A. (Biology and Psychology)

Honor or Awards: Dean's List
Graduation Honors
Sigma Xi

Publications:

Early Extracellular Events in Infection of Competent Bacillus subtilis by DNA of Bacteriophage SP82G. P.N.A.S. 69, 000-000 (1972) (in press).

Positions Held Dates
NIMH Research Trainee, Lehigh Univ. 1/69 - 6/69
Alumni Fellow, Univ. of New Hampshire 9/69 - 6/72