A REVIEW OF TRANSFORMATION IN

BACILLUS SUBTILIS

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A REVIEW OF TRANSFORMATION IN

BACILLUS SUBTILIS

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CHAPTER I

EARLY STUDIES OF TRANSFORMATION

In the process of transformation an exogenous segment of DNA from a genetically different bacterial strain is taken up and incorporated into the cell's genome. The cell then exhibits the phenotypic characteristics carried on the newly acquired DNA segment. Bacterial transformation was first reported by Griffith in 1928 (1) when he observed the death by pneumonia of mice injected with living, avirulent pneumococci and heat-killed virulent pneumococci. The mice succumbed to the type of pneumococci identical to the dead virulent organisms given by injection. In subsequent work, this pneumococcal type transformation occurred *in vitro* (2) and could be induced by a cell-free extract of the virulent organism (3). These startling discoveries began the search for the agent capable of inducing genetic transformation.

In a study lasting over ten years, Avery, MacLeod and McCarty (4) finally determined the agent to be deoxyribonucleic acid (DNA). At this time no function had been found for nucleic acids although their universal distribution in living cells was known. Saline solutions of the transforming principle in concentrations of 0.5 to 1.0 mg/ml were found to be colorless, clear and highly viscous. The purified fraction could be stored in saline solutions for several months without loss of activity. Crude extracts withstood heating to 65° for 30 to

60 minutes, but purified fractions were somewhat less heat stable. The transforming principle was rapidly inactivated at pH 5 and below. In concentrated solution the purified material gave negative results in the biuret and Millon tests, faintly positive results in the orcinol test for RNA, and a strongly positive diphenylamine test for DNA. Repeated alcohol and ether extraction or chloroform treatment had no effect on the transforming activity. Chemical analysis of the transforming principle showed an average nitrogen-phosphorous ratio of 1.67 which indicated little or no contamination by protein or other nitrogen or phosphorous containing substance. The transforming substance had little or no serological reactivity to Type III antipneumococcus rabbit serum, which detects pneumococcal protein, and capsular and somatic polysaccharides. A purified fraction showed only one sharp band following ultracentrifugation, indicating homogeneity and uniform size. Electrophoresis also showed only one component. The material had a maximum ultraviolet (UV) absorption at 2600 Å and a minimum at 2350 Å. One part in 6 x 10^8 or 0.003 µg of the purified fraction was capable of inducing transformation. Certain preparations of dog intestinal mucosa, pneumococcal autolysates, and normal dog and rabbit serum destroyed the transforming activity. Each preparation contained a depolymerase for deoxyribonucleic acid. The enzymatic action was inhibited by sodium fluoride. Trypsin, chymotrypsin, and RNase had no effect on the transforming principle, but treatment with DNase, an enzyme capable of specifically depolymerizing DNA, led to rapid inactivation. Although Avery, content with publishing only the facts of discovery, made no claims and did no theorizing, he fully realized the implications and significance of the discovery (5). And because of his reticence, many

investigators failed to recognize this cornerstone of molecular biology and later questioned whether Avery himself foresaw the importance of the discovery.

Since 1945 the search for more knowledge of the properties of DNA, the correlation between its structure and genetic function, and the quest to explain the mechanism of transformation have progressed continuously and have resulted in vast amounts of literature. Transformation has been found in several species of genera other than *Pneumococcus* (Table I). Only the initial reports of transformation in a species appear in this table.

Intergeneric transformation occurs between *Streptococcus* and *Diplococcus* (6) and between *Streptococcus* and a strain of *Staphylococcus* (28). In addition, interspecific transformation has been noted for *Hemophilus* sp. (29, 30), *Neisseria* sp. (31), *Rhizobium* sp. (18), *Pseudomonas* sp. (23), *Streptococcus* sp. (32), and *Bacillus* sp. (33, 34). An unconfirmed RNA-mediated transformation in *B. subtilis* to penicillin resistance has been reported (35). This review will deal only with the *B. subtilis* transformation system.

TABLE I

BACTERIAL SPECIES IN WHICH TRANSFORMATION HAS BEEN REPORTED

Species	Genetic Characters Involved	Reference
Diplococcus pneumoniae	Synthesis of type-specific capsular polysaccharide	Griffith (1)
Streptococcus viridans	Streptomycin resistance	Bracco, Krauss, Roe, MacLeod (6)
Streptococcus sbe	Streptomycin resistance	Pakula, Fluder, Hulanicka, Walczak (7, 8)
Streptococcus, serological group H, hemolytic strain Challis	Streptomycin resistance	Pakula, Hulanicka, Walczak (7, 8)
Hemophilus influenzae	Synthesis of type-specific capsular polysaccharide	Alexander, Leidy (9)
Hemophilus parainfluenzae	Streptomycin resistance	Leidy, Hahn, Alexander (10)
Hemophilus suis	Streptomycin resistance	Leidy, Hahn Alexander (10)
Neisseria meningitidis	Synthesis of type-specific capsular polysaccharide	Alexander, Redman (11)
E. coli	Synthesis of type-specific capsular polysaccharide	Boivin, Vendrely, Lehoult (12)
	Ability to synthesize vitamin B_{12} and methionine	Avadhani, Mehta, Rege (13)
Agrobacterium tumefaciens	Ability to induce crown-gall tumors in tomato	Klein, Klein (14)
Agrobacterium radiobacter	Ability to induce crown-gall tumors in tomato	Klein, Klein (14)
Agrobacterium rubi	Ability to induce crown-gall tumors in tomato	Klein, Klein (14)
Xanthomonas phaseoli	Polysaccharide production (colony morphology)	Corey, Starr (15, 16).

TABLE I (CONTINUED)

Species	Genetic Characters Involved	Reference
Bacillus subtilis	Ability to synthesize indole, anthranilic acid, nicotinic acid	Spizizen (17)
Rhizobium sp.	Ability to form nodules on alfalfa	Balassa (18)
Staphylococcus aureus	Streptomycin resistance	Imshenetskii, Perova, Zaitseva, Belozerskii (19)
Micrococcus radiodurans	Streptomycin resistance	Moseley, Setlow (20)
Moraxella bovis	Streptomycin resistance	Bovre (21)
Moraxella nonliquefaciens	Streptomycin resistance	Bovre (21)
Pseudomonas fluorescens	Streptomycin resistance	Lambina, Mikhailova (22)
Pseudomonas aeruginosa	Ability to synthesize isoleucine and valine	Khan, Sen (23)
Pasteurella novicida	Streptomycin resistance	Tyeryar, Lawton (24)
Micrococcus lysodeikticus	Mitomycin C resistance	Okubo, Nakayama (25)
	Mitomycin C and erythromycin resistance	Mahler, Grossman (26)
Mycoplasma laidlawii	Streptomycin resistance	Folsome (27)

CHAPTER II

B. subtilis TRANSFORMATION SYSTEMS

Discovery by Spizizen

The discovery by Spizizen (17) of transformation in *B. subtilis* provided an ideal system since this organism could grow and be transformed in a simple, chemically defined medium. The phenomenon occurred in various mutant forms requiring amino acids. Spores of the indole-requiring *B. subtilis* 168 were incubated with DNA from *B. subtilis* 23 and transformed to indole independence. Transformation was inhibited by 2 µg/ml or more of DNase with Mg^{++}. DNA from *E. coli* or strain 168 was inactive, while as little as 2×10^{-3} µg/ml of strain 23 DNA produced transformation. The number of transformants was a function of the amount of DNA used. DNA from transformed 168 cells was about ten times more effective in the second transformation than was DNA from strain 23. Spontaneous reversion to non-requirement has not been observed with the 168 strain. Transformation was also found in *B. subtilis* strain 166 (anthranilic acid$^-$, indole$^-$, tryptophan$^-$) and in strain 122 (nicotinic acid$^-$). Although transformation occurred with 2 µg/ml DNA in glucose-minimal medium, only 0.002 µg/ml DNA was required for transformation with the addition of 0.01% yeast extract, which could not be replaced by the vitamins or ribonucleotides found in yeast. Bovine serum albumin was inhibitory even in the presence of yeast extract. Spizizen also noted that removal of protein from the DNA reduced the

the minimal concentration necessary for transformation from 100 to 1,000 fold, presumably by reducing the size of the molecule entering the cell. Complete removal of RNA, which may be a specific DNase inhibitor, lowered the biological activity about 10 fold. The transforming activity was very stable in 2 M NaCl at 4° but was extremely unstable in 0.1 M NaCl. Slow freezing and thawing in 2 M NaCl reduced the activity by 99.9%, but heating to 70° for 30 minutes had no effect. B. subtilis transformation was soon confirmed and other laboratories joined in the investigations.

Competence Development

As a necessary condition for transformation, the cell must exist in an ill-defined physiological state known as competence which lasts for 3 to 4 hours (36, 37). The development of competence is considered the initial phase of transformation since maximum uptake of exogenous DNA or transfection by viral DNA occurs concurrently with maximal competence. Both competent and noncompetent cells are transduced with equal frequencies for genetic markers requiring recombination. This indicates that competent and noncompetent cells alike are capable of integration and recombination, but only competent cells take up significant amounts of exogenous DNA (38).

Isolation of stable, noncompetent mutants lacking the ability to bind DNA at any stage of growth from competent strains provided evidence that development of competence was under genetic control (39, 40). Anagnostopoulos and Spizizen (41) found competence developed near the end of the logarithmic growth phase and required L-tryptophan or indole and a cupric ion chelator such as L-histidine. The presence

of large amounts of amino acids, such as contained in acid hydrolyzed casein, reduced competence by allowing synthesis of excess cell wall components. The regimen used consisted of growing the cells at 37° in tryptose blood broth for 26 hours, transferring to liquid minimal medium containing 0.5% glucose, 50 µg/ml L-tryptophan, and 0.02% acid hydrolyzed casein, and incubating at 37° for 4 hours with shaking. The culture was centrifuged at 3500 rpm, and diluted 10 fold in fresh minimal medium containing 0.5% glucose, 5 µg/ml L-tryptophan, 0.01% acid hydrolyzed casein, and 5 umoles/ml $MgSO_4$. DNA was added, and the mixture incubated 90 minutes before diluting and plating. The maximum percent of transformation reported was 3.5 as calculated by the equation:

$$\% \text{ transformation} = \frac{\text{No. of } I^{+} \text{ transformants}}{\text{total cell count}} \times 100$$

These investigators postulated an enzyme mechanism involved in active DNA uptake based on the observed inhibition of uptake by certain metabolic poisons. Farmer and Rothman (42) found that thymine-requiring mutants of strain 168 were efficient as either donors or recipients in transformation. Horvath (43) observed optimal multiplication of B. subtilis in nutrient medium at pH 6.8 to 7.0 but no transformation when tryptophan was absent from the system, which indicated a protein synthesis requirement. Horvath (44) confirmed Spizizen's findings that a minimal-glucose-yeast (MGY) medium gave a greater percent transformation than did minimal medium. The number of transformants increased approximately linearly with increasing concentrations of yeast extract. The numbers ranged from 50 transformants without yeast extract to 800 with 3.2% yeast extract. Aeration facilitated competence development, and a reduction in the supply of oxygen halted competence

development. The average optimal generation time for competence development was 45 to 60 minutes which indicated that most of the cells were not actively growing. A closed culture, one in which the medium is not replenished or changed during growth, developed competence when the growth medium was exhausted of some nutrients, and competence increased much faster than did the growth rate. However, a culture in balanced growth with fresh MGY medium added every 10 minutes maintained an approximately constant optical density and remained at the same level of competence for long periods. Basic amino acids such as lysine, arginine, and histidine at 200 µg/ml enhanced competence development in an unspecified species of Bacillus (45). Young and Jackson (46) showed that teichoic acid which contaminated isolated DNA did not influence competence development or the incorporation of DNA and did not enter the recipient cell during competence. The teichoic acid was soluble in perchloric acid and could be separated from the DNA on agarose or methylated albumin keiselguhr columns. Stewart (47) reported transformation five times as great in Spizizen's minimal medium without the customary 0.14 M potassium phosphate as in the normal minimal medium in the linear response range, but at saturating DNA levels the increase was insignificant which suggested that phosphate may be an inhibitor of transformation. Low levels of transformation were obtained without Mg^{++}, but its addition increased the transformation frequence. The optimal concentration of Mg^{++} was well above the 0.001 M cited by Spizizen. With 0.02 M $MgCl_2$, the transformation frequency increased 2 to 4 fold over that obtained with Spizizen's minimal medium. Addition of $MgCl_2$ to Spizizen's minimal medium created a white precipitate which was

presumed to be the phosphate precipitated by Mg^{++} since this phenomenon did not occur in the phosphate-free medium. Addition of β-mercaptoethanol to minimal medium increased the transformation up to 2 fold in the linear range of DNA, with an optimal β-mercaptoethanol concentration of 8×10^{-4} M. Apparently the β-mercaptoethanol stimulated transformation itself as well as reacting with cupric ions. Acridine orange, which complexes with DNA, also inhibited B. subtilis transformation; and phleomycin, which inhibits DNA synthesis, added at a concentration of 0.2 μg/ml at the time of DNA addition caused a 10-fold transformation reduction and 40% viable cell loss. Table II shows a summary of factors influencing the development of competence.

Although protein synthesis is required in the development of competence (36, 48), inhibition of DNA synthesis by thymine starvation did not affect competence formation in a thymine-requiring B. subtilis strain (42). This evidence agreed with that of Bodmer (49) who demonstrated competence development and transformation when DNA synthesis was as low as 0.15% of the normal synthetic activity. McCarthy and Nester (50) demonstrated 40 to 50% of the competent cells in a culture to be latent for DNA synthesis while the remaining cells synthesized DNA at about half the normal rate. Protein synthesis occurred although stable RNA synthesis did not. This biosynthetic latency may last for 2.5 to 3 hours. Newly transformed cells showed similar biosynthetic patterns, in that they were unable to carry out several different synthetic reactions including at least one or more concerned with growth and multiplication (51). Nester and Stocker (51) found that even though this general state of competence may persist several hours, recombination occurred with 30 minutes of DNA

TABLE II

FACTORS INFLUENCING THE DEVELOPMENT OF COMPETENCE

Enhancers	Inhibitors
L-tryptophan or indole (41)	High concentrations of amino acids (41)
L-histidine (41)	Reduction in oxygen supply (44)
Yeast extract (17)	Periodate (88)
Aeration (44)	Phenethyl alcohol (89, 90)
Lysine, arginine and histidine (45)	
Mg^{++} (47)	
Thalidomide (91)	

addition. They estimated the competent fraction of the culture to be 1 to 5% of the total by applying the equation:

$$\% \text{ competent cells} = \frac{(\text{frequency tryptophan}^+)(\text{frequency histidine}^+)}{(\text{frequency histidine}^+\ \text{tryptophan}^+)} \times 100$$

Nester (36) noted competent cells were more resistant to penicillin, which preferentially kills growing cells, and concluded that competent cells were not growing or multiplying. A stylized growth curve (Figure 1) indicates the relationship between growth and transformation.

Kammen, Wojnar, and Canellakis (52) reported competence development and maintenance in B. subtilis required continued synthesis of a protein mediated by m-RNA. They suggested the extended period of competence coincided with the lag in transformant replication, the lag in phenotypic expression, and with antibiotic resistance of potential transformants. Archer and Landman (53) found development of competence in nondividing cells without net DNA synthesis or chromosome replication. Following amino acid starvation, the addition of thymine and amino acids initiated DNA replication while amino acid addition alone was sufficient to permit synthesis of the protein(s) for competence development. The chromosomes of competent cells were arrested by amino acid starvation just as were those of noncompetent cells.

Competent cells are less dense than the bulk population of a culture of B. subtilis (54, 55, 56) and shorter than the average noncompetent cell (57). Singh and Pitale (58) suggested that uninucleate cells are more likely to be competent than are multinucleate cells. Okubo and Romig (59) showed competent bacteria to differ from noncompetent cells in their capacity for repair of UV-induced lesions in

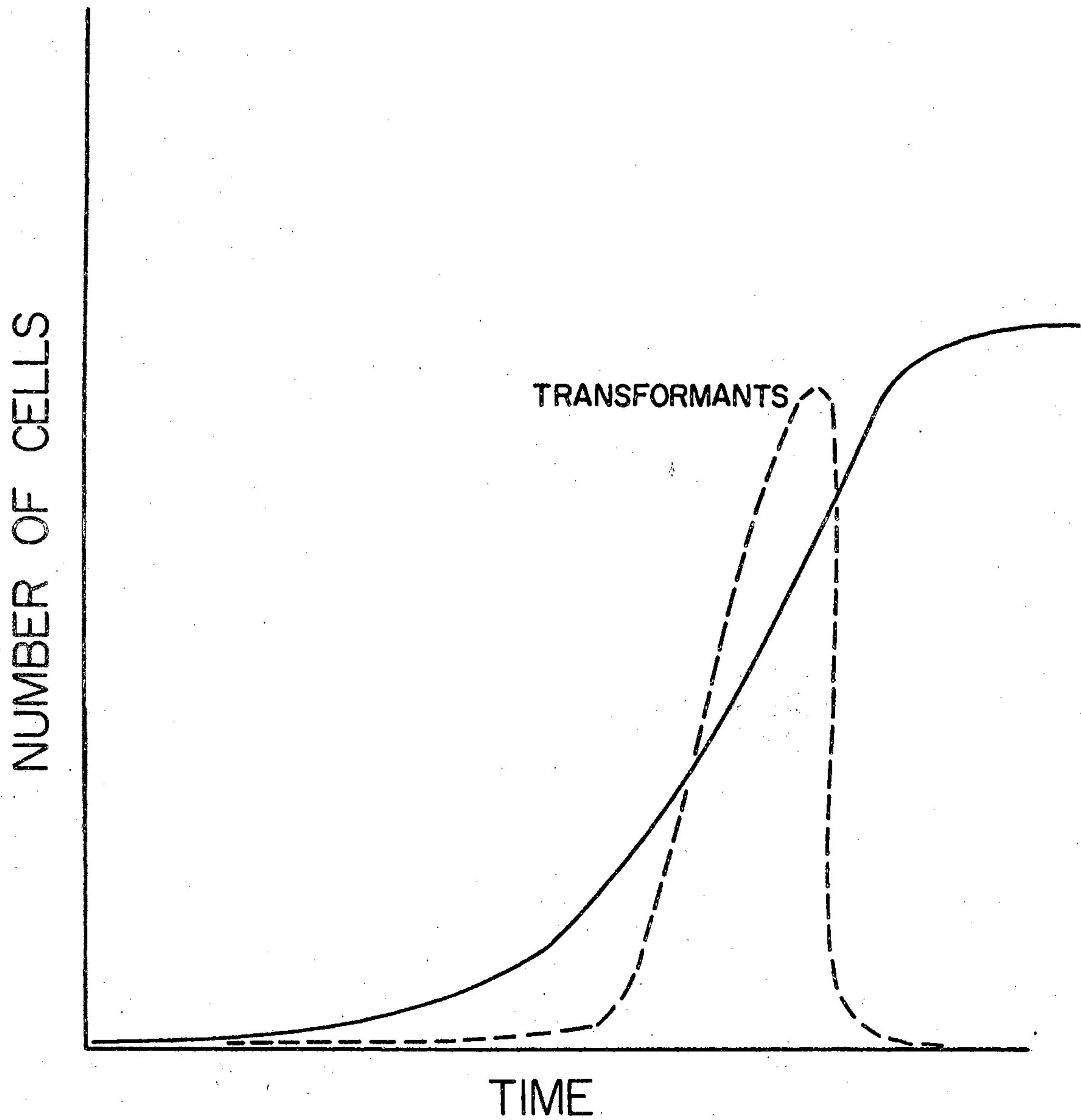


Figure 1. Stylized Growth and Transformation Curves

transforming DNA, and attributed this to the biosynthetic latency of the competent cell although DNA alterations interfering with repair were not entirely ruled out. Kelner's work (60) indicated little or no photoreactivation in the competent, transformed fraction of a B. subtilis culture. This was a temporary condition, probably due to a specific enzyme deficiency during biosynthetic latency, and nonphotoreactivability was the only aspect common to the known transformable bacteria at the time of this study. Jensen and Haas (61) studied the stable and regular electrokinetic patterns of B. subtilis cultures and showed cells become competent at a time when the electrokinetic potential reached a minimum and charge density was presumed to reach a maximum. These workers also reported (62): 1) direct relation of physiological competency to electrical charge on the cell surface; 2) newly competent cells carry a maximum negative charge which gradually decreases afterwards; 3) newly competent cells appear with spontaneous abruptness; 4) a kinetic flow from highly charged to lower indicates progressive surface charge loss by the competent cell; 5) competent cells undergo transformation over a range of surface charge.

Competence in B. subtilis and the capacity for sporulation have been associated (40, 63). Involvement of the fore-spore membrane in transformation has been implicated by both Young (64) and Spizizen, Reilly and Evans (37). However, nonsporulating mutants of B. subtilis which retain their competence have been found as well as noncompetent, nonsporulating mutants (65). Spizizen (66) reported the possibility of at least three sporulating loci in B. subtilis and cited conditions for optimal transformation as growth at 37° for 5.5 hours or more in

minimal medium plus the growth factor, indole or L-tryptophan, and a low concentration of acid hydrolyzed casein. More recently, Balassa (67) estimated the number of genes involved in sporulation at about 800. Copper was thought by Weed (68) to be important in metabolism and to affect sporulation since exposure of a tryptophan-requiring auxotroph of B. subtilis 168 to copper led to the isolation of a similar, but nontransformable, strain and a small-colony strain differing in DNA biological activity and chemical composition from the parental strain. Ryter (69) has described six successive stages in sporulation which were characterized by the appearance of nuclear and cytoplasmic material and by the number and aspect of spore envelopes when studied in both light microscopy and electron microscopy. Stage I is designated as elongation of the nucleus, which may be in contact with one or two mesosomes, and formation of a protruberance on the inner cell wall which will become the septum. In stage II the nucleus is reduced in size and a full septum forms. Deformation of the septum and organization of an intracytoplasmic, ovoid-fore spore whose envelope consists of two membranes constitutes stage III. The mature spore becomes heat resistant in stage IV, and between t_3 and t_5 a cortex develops consisting of an outer electron dense layer and an inner clear layer. In stage V more coats form around the spore and it becomes resistant to octanol. The cortex has attained about half its final thickness by $t_{5.5}$ and continues to thicken during stages V and VI. Stage VI consists of cytoplasmic changes. The stages of sporulation in B. subtilis and corresponding time intervals in hours appear in Figure 2. Sterlini and Mandelstam (70) also reported the existence of successive steps in sporulation including alkaline phos-

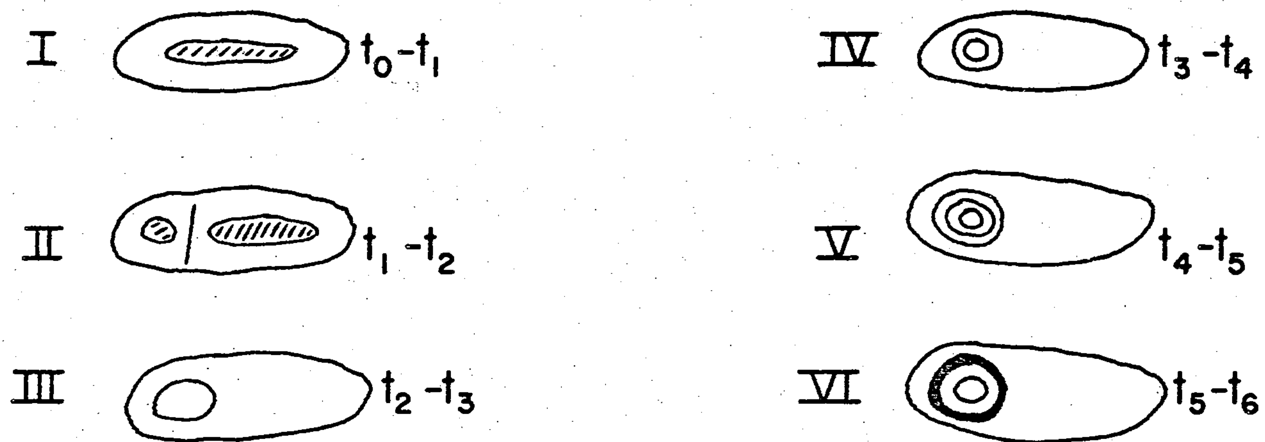


Figure 2. Stages of Sporulation (69)

phatase appearance, refractility, dipicolinic acid formation, and heat resistance. Each step became refractive to actinomycin D about 30 minutes after commitment. This study indicated that sporulation was regulated at the level of transcription and translation.

Function of Cell Wall and Mesosome

Attention was next focused on the cell wall as the limiting condition in competence formation when Spizizen (71) found that development of competence required a period of unbalanced growth and was inhibited by excessive amounts of cell wall precursors. However, competence has developed in cultures without a period of unbalanced growth and without the customary medium shift (44). Young, Spizizen and Crawford (72) and Young (73) noted a difference in the cell wall composition of competent and noncompetent cells; namely, the walls of competent strains contained more galactosamine and alanine while noncompetent strains had more glutamic acid, diaminopimelic acid, and glucosamine. Values obtained with competent (C^+) and noncompetent (C^-) cells of strain 168 indole$^-$ (I^-) are compared (Table III), and a quantitative chemical change in the cell wall of the transformable strain during growth is seen. The numbers in parenthesis designate the hours in growth medium (72). As expected, galactosamine decreased when the transformable strain was grown in more complex medium which inhibited competence (73). Young, Spizizen and Crawford (72) also postulated that competence required the development of specific DNA adsorptive sites and was dependent upon an alteration of the cell surface. Young (64) suggested that galactosamine, which is at a maximum concurrently with maximum competence, may attach to the

TABLE III

CELL WALL COMPOSITION IN COMPETENT AND NONCOMPETENT CELLS

Compound	168 $I^-C^+(1\frac{1}{2})$	168 $I^-C^+(5)$	168 I^-C^+ (12)	168 $I^-C^-(5)$
	moles/2 mg hydrolyzed cell walls			
Alanine	1.50	1.50	1.53	1.27
Diaminopimelic acid	0.73	0.77	0.89	1.03
Glutamic acid	0.62	0.63	0.73	0.77
Muramic acid	0.56	0.57	0.60	0.68
Glucosamine	0.68	0.67	0.65	0.81
Galactosamine	0.44	0.43	0.39	0.25
Ammonia	1.17	1.24	1.44	1.22

terminal phosphorous groups, thereby decreasing the net negative charge on the cell wall and facilitating penetration of the negatively charged DNA.

Autolytic Enzyme

Young and Spizizen (74) reported a heat-labile, nondialyzable enzyme in B. subtilis cell walls capable of degrading the wall. The enzymatic activity was greatest in transforming strains before and at the time of maximum competence and was implicated in the synthesis or degradation of the cell wall during the logarithmic growth phase but not in presporulation and sporulation. The rate of lysis in poorly transformable strains was 3 to 4 fold lower than in the highly transformable strain, and the heteropolymers produced by cell wall degradation in the transformable strain were different from those of the non-transformable strains. Young, Tipper, and Strominger (75) assigned the autolytic enzyme to the acetylmuramyl-L-alanine amidase group, and suggested as its function localized hydrolysis resulting in the relaxation of the glycopeptide structure to permit DNA entry into the cell. Young (76) found cell walls from early exponential growth phase to autolyze more rapidly than walls from the stationary phase apparently due to intrinsic differences in crude enzyme activity. Synthetic substrates for the enzyme were not identified, and the typical trypsin and chymotrypsin substrates, such as benzoyl-L-arginine methyl ester, acetyl-L-tyrosine ethyl ester, alanylglycylglycine, and alanylglycine, were not hydrolyzed by the autolytic enzyme. The rate of autolysis was dependent on temperature; linear between 24 and 45° when the log of the rate constant was plotted against the reciprocal of absolute

temperature, but above 62° complete lysis did not occur. The pH optimum of the autolytic enzyme was 9.5 while lysozyme and most other β-N-acylhexosaminidases have pH optimums below 7.0. Na^{+} and K^{+} were equally efficient in stimulating autolysis while divalent cations affected autolysis and transformation similarly, with the exception of Mn^{++} which stimulates autolysis and does not affect transformation. The enzyme exhibited first order kinetics and an energy of activation of 9.2 kcal per mole. Young (76) postulated the enzyme action to be either polysaccharide cleavage (A) or peptide chain cleavage (B through E) as illustrated in Figure 3 where R, R', and R'' are glutamic acid, diaminopimelic acid, and alanine respectively. The maximum activity of the autolytic enzyme in the log phase indicated a growth function, perhaps hydrolysis of cross-linked peptides to permit cell wall expansion and to facilitate insertion of small segments of cell wall transported through the cytoplasmic membrane on a lipid carrier or insertion of teichoic acid into the basal mucopeptide requiring a transpeptidase to synthesize peptide bonds and forming a three-dimensional cross linked structure.

Extracellular Factors

Extracellular proteins which act as competence-stimulating or -inducing factors have been identified in the following transformation systems: _Streptococcus_ (77, 78), _H. influenzae_ (79), _Pneumococcus_ (80), and _B. subtilis_ (81). The competence factors of _Streptococcus_, _Pneumococcus_, and _B. subtilis_ are sensitive to proteolytic enzymes and heat and can be inactivated by filtration through membranes, while that of _H. influenzae_ is relatively heat stable and refractive

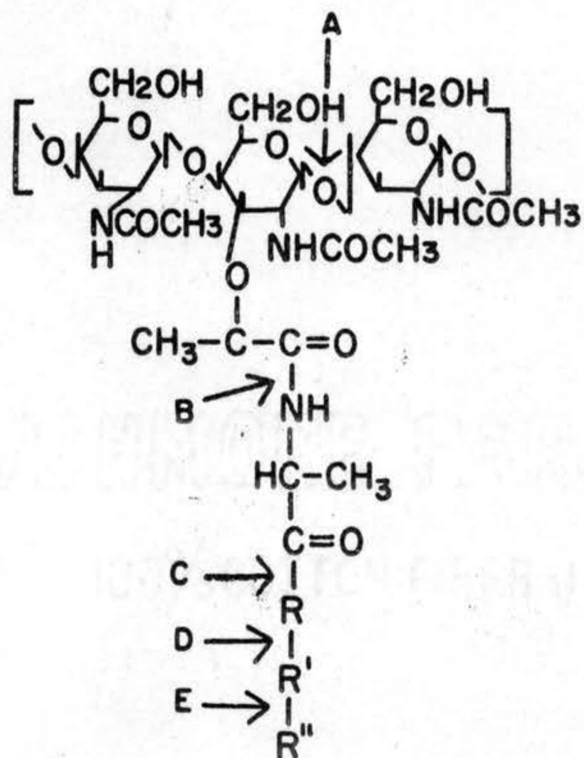


Figure 3. Sites of Autolytic Enzyme Action on Cell Wall (76)

to proteolytic enzymes, RNase, and DNase. Both streptococcal and pneumococcal competence factors carry a net positive charge in physiological pH range. The pneumococcal factor is specific for *Pneumococcus* and of relatively high molecular weight.

Felkner and Wyss (82) first reported a competence factor removable from *B. cereus* by washing with distilled water. Concentrated competence factor added back to washed cells rendered them transformable. However, work by Goldberg and Gwinn (83) indicated the strain used by Felkner and Wyss (82) was actually *B. subtilis* 168 I^-. The "*B. cereus*" of Felkner and Wyss was transformed by DNA from *B. subtilis* but not from *B. cereus*. SP10, a phage incapable of plaque formation on *B. subtilis* 168 I^-, did not produce plaques on "*B. cereus*," and the melting temperature (T_m) of "*B. cereus*" 569-S^r DNA at 85.9^o approximated that of *B. subtilis* 168 I^- at 85.5^o more closely than the 82.4^o of confirmed *B. cereus* DNA. Charpak and Dedonder (81) reported a soluble competence factor in *B. subtilis* which was destroyed by heat, trypsin, or chymotrypsin. Akrigg, Atkinson, Ayad, and Barker (84) showed that aqueous extracts of competent *B. subtilis* 168 possess competence-restoring ability when added to water-extracted cells. Pancreatic RNase had no effect on the restorative ability of this extract, but trypsin destroyed it. The extract showed five peaks on DEAE cellulose chromatography. Peak II contained the transformation-stimulating property while aqueous extracts of noncompetent cells showed only one peak which chromatographed at a different point than did peak II. Peak II was later shown capable of lysing heat-inactivated cell walls upon incubation (85). Akrigg and Ayad (85) suggested that peak II, apparently a polypeptide based on its inactiva-

tion by trypsin, may actually be the autolytic enzyme in B. subtilis cell walls. The next logical step in research along this line is the purification of the autolytic enzyme and comparison of it and peak II by chromatography.

Antibiotic Resistance

In B. subtilis the mutation to actinomycin resistance was thought to correlate with the loss of ability to become competent and with reduced sporulation ability; however, Ephrati-Elizur (86) reported an actinomycin resistant mutant which could sporulate and transform more readily than the other actinomycin resistant mutants. Fraser and McDonald (87) found spontaneously occurring streptomycin-dependent mutants by plating B. subtilis 168 on beef heart infusion agar plus dihydrostreptomycin (DSM). This mutation was about 1/10 as frequent as the mutation to streptomycin resistance. From the streptomycin-dependent strains these workers isolated at least three types of non-dependent mutants including the following: 1) those resistant to 1 mg/ml or more of DSM were termed str-d/str-r (1)-1; 2) those resistant to lower concentrations termed str-d/str-r (0.5)-1; 3) those sensitive to DSM as are the wild type were termed str-d/str-s-1. Transformation systems using a streptomycin resistant mutant $168S^r$ and each of these nondependent mutants as donors and 168 as recipients resulted in a majority of streptomycin-dependent colonies and a few streptomycin-resistant colonies indicating that streptomycin resistance is due to unlinked suppressor mutations. The streptomycin-sensitive mutants found were apparently identical to the wild type.

Effects of Chemicals and Enzymes on Competence

The effect of several chemical agents on competence in *B. subtilis* has been determined, and here we shall consider work involving periodate, phenethyl alcohol and thalidomide. Polsinelli and Barlati (88) showed periodate to reduce transformation at concentrations which had no effect on cell viability. Periodate did not act on the transforming activity of the DNA, did not affect transformation when added later than 30 minutes after DNA addition, but did reduce transformation when added to the cells before or a short time after DNA addition. Periodate reduced the cell's ability to take up DNA, and Polsinelli and Barlati (88) postulated alteration of some factor involved in the binding and/or permeation of transforming DNA. Richardson and Leach (89) noted that phenethyl alcohol (PEA) at a level which did not decrease growth or kill *B. subtilis* markedly inhibited transformation by apparent interaction with competent cells making them less competent. Urban and Wyss (90) also studied the effect of phenethyl alcohol on transformation and found PEA to render competent cells unable to incorporate irreversibly transforming DNA and to decrease transformation to less than 10% of the untreated control. They suggested the following two modes of action: 1) PEA could selectively kill competent cells; 2) PEA treatment may cause the cell to become impermeable to DNA. The second mode of action was favored, and the cytoplasmic membrane was implicated as the site of impermeability to DNA as well as the location for fixing "stored" DNA. Lorkiewicz, Dudek, and Ziemiecka (91) reported a stimulation of transformation by thalidomide which was apparently due to the drug's effect on competence rather than its mutagenic effect or a selection process

based on a nutritional effect.

Landman and Halle (92) reported that treatment of a B. subtilis 168 culture with a solution of crystalline lysozyme caused bacteria to pass through an osmotically sensitive bacillary phase to a protoplast stage. All the protoplasts were recovered as L-colony-forming elements on complex or chemically defined osmotically stabilizing agar medium. Protoplasts or L-bodies could be propagated in the L-state indefinitely or could be mass reverted to the bacillary state by altering the medium ingredients. Soft agar and several D-amino acids inhibited reversion, while hard agar and 30% gelatin media induced reversion which was often 100% effective and began within 5 hours of plating. L-forms induced by penicillin differed in their resistance to penicillin from the bacillary forms and seemed identical to lysozyme-induced L-forms. Since cells without cell walls appeared unable to reinitiate division and wall forming functions, the interpretation of Landman and Halle was that cell wall removal deprives the cell of a component of a self-sustaining reaction chain required for septation and wall-formation. Commitment to the L-colony form was complete when the wall was removed, and hence the missing component or primer was perhaps in the wall or more probably in the membrane. The nature of the primer was undetermined leaving the possibilities of enzyme, metabolic intermediate, cell wall fragment, RNA messenger or something else found in the cell. As lysozyme, which hydrolyzes the β-1,4 linkage between muramic acid and N-acetylglycosamine in the cell wall, attacked bacilli, Miller, Zsigray, and Landman (93) found cells to first pass to a rod-shaped osmotically sensitive stage, then to a spherical stage, and finally to naked protoplasts. On

medium containing D-methionine the spherical forms resulted in L-colonies which are bounded only by a membrane, are irregularly shaped, and may not contain nuclear material or may be viable. On medium without D-methionine which allowed cell wall rebuilding, however, bacillary colonies were formed.

Cell Membrane

Wolstenholme, Vermeulen, and Venema (94) suggested that the membranous bodies visible in the electron micrographs of cells produce enzymes for DNA incorporation or breakdown and utilization or expulsion of DNA not incorporated. The membranous bodies increased during maximum competence. In autoradiographs of labeled DNA and maximally competent cells the DNA was closely associated with, but still outside, the membranous bodies, especially those in the cytoplasm. Autoradiographs registered exclusively the irreversible uptake of DNA and allowed direct morphological study of competent bacteria (57). Incubation of *B. subtilis* with radioactive DNA left autoradiographic grains localized at the tips and middle of the cells establishing existence of specific sites for uptake and suggesting a special class of mesosomes localized specifically within the cell. Irreversibly bound DNA was still seen at the uptake sites 90 minutes after the addition of DNA which led to the assumption that uptake and integration occur at the same site or that integration may not yet be complete at 90 minutes. DNA uptake may also occur near a septum by participation of some organelle involved in normal cell division.

Sueoka and Quinn (95) found genetic and biochemical evidence of chromosome attachment at the origin and terminus to the cell membrane.

Genetic studies indicated the membrane fraction was enriched for adenine 16, a marker very close to the replication origin, and slightly enriched for methionine, a marker close to the chromosome terminus. Biochemical evidence was obtained through tritiated thymidine labeling of the origin of DNA which was also found in the membrane fraction. Their proposed model of DNA replication appears in Figure 4. Ryter and Jacob (96) showed direct linkage of nucleus and membrane in E. coli spheroplasts and B. subtilis protoplasts, probably through only one linkage per protoplast or spheroplast. Ryter and Landman (97) demonstrated that soon after lysozyme addition, mesosomes which are normally in contact with both the peripheral membrane and the nuclear material were extruded from the cell interior into the space between the wall and the cytoplasmic membrane. Mesosome fragments gathered at the poles and were released with the protoplast when the wall broke. With mesosome extrusion, the nuclear material became directly attached to the cytoplasmic membrane. Jacob, Ryter, and Cuzin (98) postulated that if cellular replicons are attached to the membrane where their replication occurs and is regulated, the method of DNA reproduction could conceivably be correlated with cellular growth through signals transmitted by the membrane to the enzyme system. However, since bacteria have no complex mitotic apparatus, chromosome separation and distribution may occur through growth of a membrane in the area located between the points of attachment of chromosome to membrane. Every B. subtilis nucleus was in close contact with one or two mesosomes except in the dormant spore where nucleus-membrane contact was direct. Electronmicrographs of the division cycle (Figure 5) (98) showed the nucleus first as a small structure

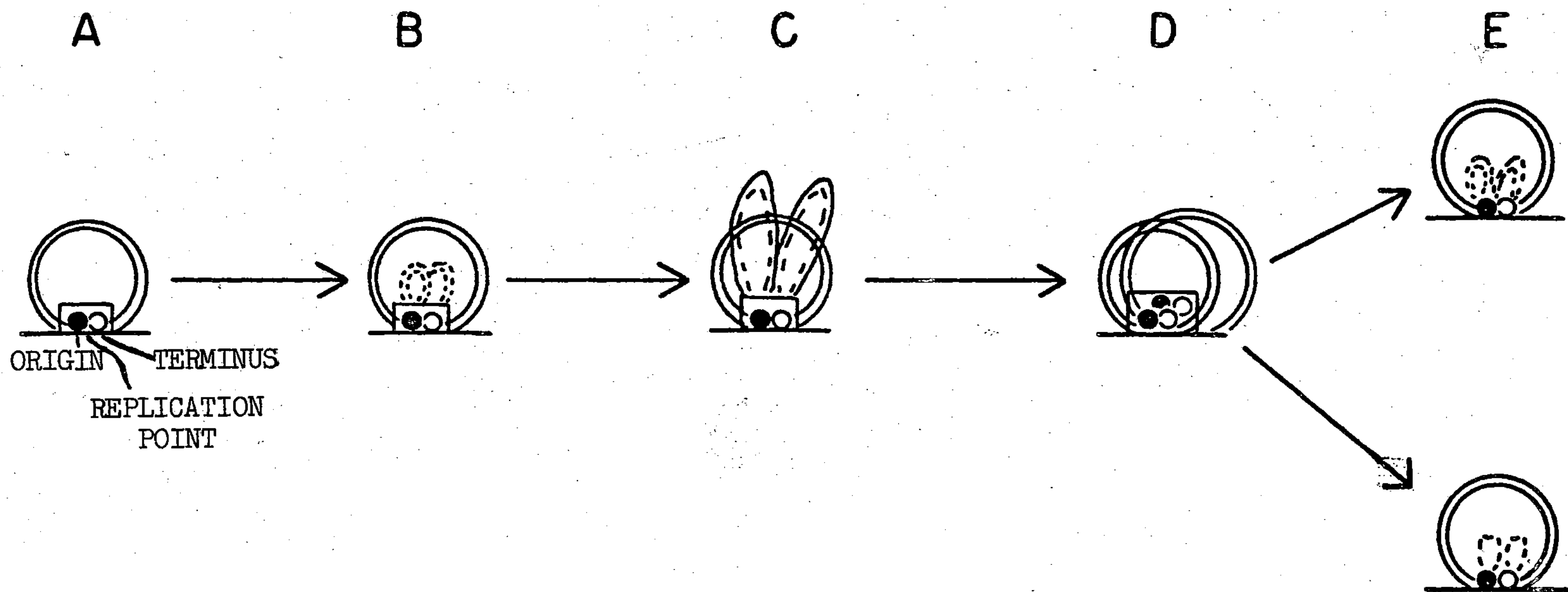


Figure 4. Chromosome Replication in *B. subtilis* (95)

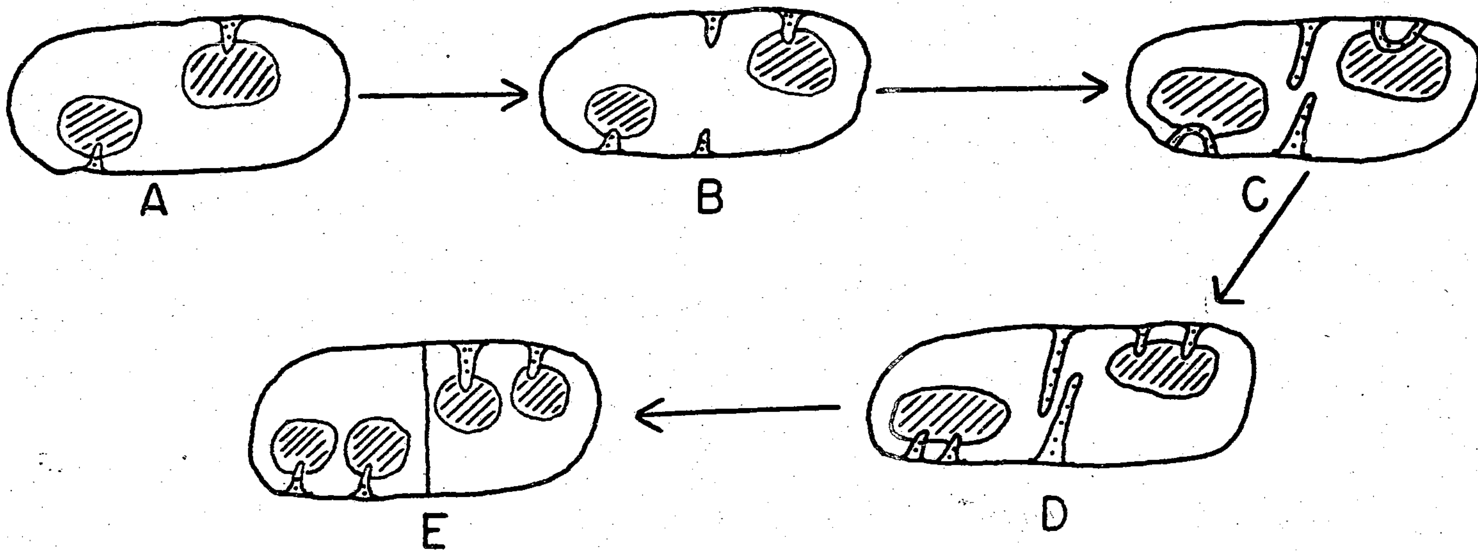


Figure 5. Chromosome Separation and Distribution (98)

attached to a single mesosome. The nucleus then increased in size, and at a certain stage was attached to two adjacent mesosomes which apparently arose from a doubling of the first. The two mesosomes moved farther and farther apart, and the nucleus separated into two nuclei, each associated with one mesosome. Membrane synthesis could not be determined but was considered possible since one or two mesosomes were always found in the septation region. The proposed model (98) leads to the following predictions: 1) the bacterial chromosome should be found connected in some way with the membrane; 2) membrane synthesis at the division cycle occurs in well-defined regions; 3) old fragments of membrane should segregate with old DNA chains; 4) the replicating fork of DNA should be associated with the membrane since the model suggests a slow revolution of the DNA structure during replication with the enzyme complex incorporated into the membrane; 5) the enzyme system for replication should be found in the membrane.

A recent study (99) showed that complete cell wall removal makes the cell refractory to donor DNA, but that quasi-spheroplasts, spheres with adherent cell walls, of B. subtilis transform if plated on medium which allows continuance of cell wall synthesis after DNA addition. Thus DNA transport may require the presence of mesosomes. Transformation inhibition found in quasi-spheroplasts indicated two successive steps in DNA uptake——uptake into the cell periphery, and transfer into the cell's interior. The lack of transport to the interior suggested eversion of mesosomes in quasi-spheroplasts unable to rebuild their cell walls. Akrigg, Ayad, and Blamire (100) have suggested a mechanism for DNA uptake (Figure 6) by competent B. subtilis

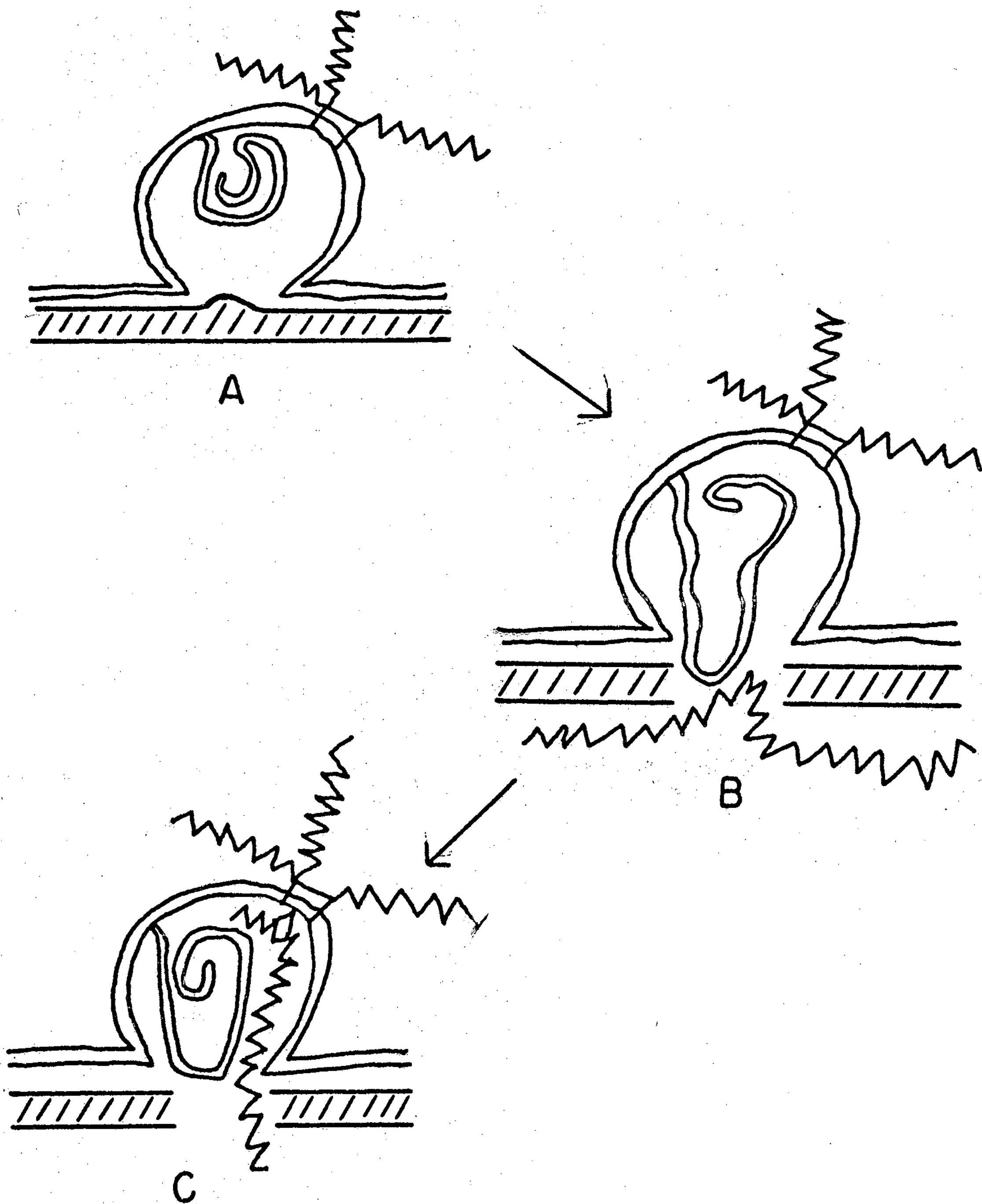


Figure 6. Mechanism of DNA Uptake in Competent B. subtilis (100)

with the following stages: 1) a lytic enzyme, usually localized in the cell wall, is released and attacks the cell wall in the area of a point of weakness; 2) one of these areas would be next to a mesosome; 3) cell wall rupture exposes the mesosome to the outside medium where DNA may associate with it; 4) DNA is then taken up into the mesosome and reaches the recipient DNA at a replicating point, thus requiring the donor DNA to pass through only one intact membrane, that of the mesosome.

CHAPTER III

TRANSFORMING DNA

Isolation

Marmur (101) reported a DNA isolation procedure successfully used in many bacterial species which consisted of cell lysis with sodium dodecyl sulfate (SDS), chloroform-isoamyl alcohol treatment, repeated precipitation with ethanol and deproteinization with a chloroform-isoamyl alcohol solution, RNase treatment, precipitation with isopropyl alcohol, and purification in ethanol. Sevag (102) demonstrated another DNA isolation procedure which included cell lysis by lysozyme in a phosphate-sucrose buffer, successive extractions with 10% NaCl plus 1% trisodium citrate, repeated chloroform-octanol deproteinization, RNase treatment, further deproteinization, and precipitation with ethanol. Since the early deproteinizing methods were, of necessity, repetitious and caused loss of biological activity, Saito and Miura (103) applied the more gentle phenol treatment which had previously been used in DNA isolation from phages (104, 105). The comparative activities and cotransfer indices of DNA isolated by the above methods are shown in Table IV (103).

Effects of Physical Modification of Transforming DNA

Fragmentation or Shearing

TABLE IV

BIOLOGICAL ACTIVITY OF DNA ISOLATED BY VARIOUS METHODS

Method	No. Transformants ($x 10^{-2}$/ml)			Cotransfer Index
	I^+	H^+	I^+H^+	
Sevag	144	120	62	0.31
Marmur	225	204	127	0.42
Phenol pH 6	578	499	248	0.30
pH 7	1388	975	877	0.59
pH 8	838	702	502	0.48
pH 9	1373	1170	976	0.62

Thymine starvation was shown to produce a very fragile DNA which was partially degraded by the thermal or enzymatic instability of the incomplete double helix, by mechanical forces during replication after thymidine addition, or by shear forces during deproteinization (106). Although the entire B. subtilis genome is present in a solution of isolated DNA, it may be broken in 50 to 200 pieces with an average molecular weight of 1×10^7 daltons, depending on the isolation procedure used (107). In this molecular weight range degradation requires stronger shearing forces, such as exposure to sonic waves or passage through an atomizer, than those present in mere handling of the solution. The effect of such shearing is a clear-cut scission of the double helix which unlinks loosely linked markers but does not affect UV absorption or T_m. Molecular size has been implicated in the efficiency of cotransfer of unlinked markers by Spizizen, Reilly, and Evans (37) who showed that the cotransfer index of methionine and indole markers in a DNA preparation was reduced to 27% of the control when forced through a 27 gauge needle at a flow rate of 2 ml/minute. Nester, Ganesan and Lederberg (108) found the highest molecular weight fraction of extracted DNA the most likely to be integrated, while mechanical shearing of the DNA resulted in lower sedimentation values, reduced viscosity, and decreased genetic complement. The physical properties of sheared DNA suggested molecules about half the original size of extracted DNA. Transforming activity, intrinsic viscosity, and molecular weight of DNA were unchanged after filtration through cellulose ester filters, thereby providing a quick and efficient method for dust removal from DNA used in light scattering measurements (109).

Dense Form of DNA

Harris and Barr (110) observed the accumulation of an unusually dense form of DNA in which about 5%, at most, of the bases were present as the single-stranded form. Transforming DNA entering a competent cell was quickly converted to this partially single-stranded form, which was suggested as a basis for the specific association between donor and recipient DNA. The proportion of dense DNA increased with the competence level (111). Transforming DNA recovered from competent, transformed cells was quickly converted into a dense form similar to that in competent cells not previously transformed. Harris and Barr (111) suggested the following explanations: 1) transforming DNA is soon converted to partially single-stranded DNA and gradually converted to native DNA upon host association while the second chain is destroyed during or after association; 2) transforming DNA, which may be of any configuration in association, associates with the dense form in competent cells and is repaired to native DNA with the destruction of the second chain.

Base Analogues

Bodmer and Grether (112) noted the low efficiency of incorporation of thymine in *B. subtilis* and suggested that the low efficiency of incorporation of thymidine might be due to its rapid conversion to thymine by thymidine phosphorylase. These workers found from 55 to 95% of the uracil, uridine, and 5-fluorouracil added to a culture incorporated within the acid-insoluble fraction of *B. subtilis* SB 19 and 503. Thymine was incorporated to less than 1% and thymidine to less than 12%. With the exception of thymidine, none of the above-

mentioned compounds were found in the cell's DNA. Ephrati-Elizur and Zamenhof (113) noted that DNA containing 5-bromouracil (BU) still retained transforming activity, and Ephrati-Elizur, Zamenhof, Szybalski, Opara-Kubinska, and Lorkiewicz (114) later showed biological activity in DNA even after extensive labeling with BU in one or both strands. Opara-Kubinska, Lorkiewicz, and Szybalski (115) reported highly preferential sensitivity to irradiation introduced into the DNA by exposure to 5-bromodeoxyuridine (BUdR). Hologenation of the DNA sensitized both intact cells and extracted DNA to about the same extent, and DNA became the principal target of lethal radiation effects. Gimlin, Farquharson, and Leach (116) reported a reduction in the total cell count when BU-labeled DNA was used in transformation although acid hydrolysis of the BU-labeled DNA abolished the growth inhibition effect and addition of free bromouracil did not inhibit growth. Further work in the same laboratory showed uptake of homologous, but imperfect DNA, in this case highly substituted with bromouracil, followed by integration resulted in lethal transformation, activation of a nonlytic but lethal prophage, or interference with recombination (117). Intact BU-containing DNA was found necessary for the killing of cells, and biological specificity was involved since *E. coli* DNA labeled with bromouracil was not lethal. Competence was also found necessary for killing. Killing and transformation had similar kinetics when the latter was terminated with DNase.

UV Irradiation

The effects of UV light on transforming DNA was another facet of study, and the results of "selfing" experiments by Jensen and Haas (118)

suggested that since transforming DNA contained no induced mutations until after post-irradiation DNA synthesis began, the primary action of UV light was the production of radiochemical reactants leading to a cellular pool of modified nucleic acid precursors. Mutation production appeared confined to the initial post-irradiation doubling of DNA. Zamenhof and Reddy (119) found spores of B. subtilis more resistant to UV light in high doses, and irradiation of spores often avoided lysis caused by prophage induction in vegetative cells. Compared with vegetative cells, spores had a higher mutation frequency at the same survival level. The infliction of a primary mutational injury was possible in the absence of DNA replication. UV-induced mutants reached a plateau while the mutation rate increased with dosage of ionizing radiation. The repair mechanism for UV damage might be different at different levels of survival. Sulfanilamide resistant mutants were less resistant to ionizing and UV radiation, which suggested a faulty repair system since lesions in these mutants were not thymine dimers. Wacker, Menningmann, and Szybalski (120) noted that the thymine substitute, bromouracil, rendered DNA more UV sensitive than the normal DNA, even though it does not dimerize. This sensitivity is apparently due to instability and photochemical dehaolgenation resulting from the energy transfer and interaction between the closely neighboring halogen and phosphate groups. Opara-Kubinska, Kurylo-Borowska, and Szybalski (121) soon confirmed the greater sensitivity to UV light of BU-labeled DNA which they found also reflected by the following: 1) increased affinity of UV-treated BU-labeled DNA for a methylated albumin column; 2) decreased density and pronounced band spreading in density gradient runs; 3) marked

cross linking of complementary strands in BU-labeled DNA. Transformation frequency and resistance to UV inactivation were correlated when markers on the G-C rich molecules were found more UV resistant, and base difference was suggested as the determinant of transformation frequency (122). Mahler (123) found B. subtilis cells capable of dark repair but observed little, if any, repair to intracellular transforming DNA. Since competent cells showed no increased UV sensitivity, the lack of dark repair might be due to a secondary structure of the DNA, such as single-strandedness.

Tritium Decay

McCarthy and Nester (124) showed tritium-labeled thymidine and tritiated amino acids to yield parallel results with regard to both viability loss and gene inactivation suggesting that the lethal effect is independent of the macromolecule containing tritium. Thus, they attributed death from tritium incorporation to the ionizing radiation inactivation of the genome.

Fractionation

Saito and Masamune (125) succeeded in fractionating DNA according to base composition on a methylated albumin column. Ayad, Barker and Weigold (126) also fractionated native B. subtilis DNA and showed the transforming activity confined to two of the four fractions while denaturation at 100° yielded five fractions, two of which contained residual activity. "Redenaturation" at 100° interconverted four of the five fractions, and "redenaturation" with labeled ^{15}N and ^{2}H DNA suggested a specific component which did not take part in the inter-

conversions. Partial separation of the arginine and histidine-tryptophan markers was obtained. Rudner, Lin, Hoffman, and Chargaff (127) showed inactivation of DNA by dialysis proportional to the duration and DNA concentration. Interactions between DNA molecules in highly concentrated solutions undergoing dialysis enhanced the denaturing effect. After dialysis, electrolyte addition led to partial reactivation providing dilution had not previously occurred. Recently Ayad (128) reported that the residual biological activity in DNA after heating to 100° could be due to cross links or to a specific G-C content. A sharp drop in biological activity was observed at first, with a slower decrease afterwards which indicated complete unwinding of the double helix. The apparent residual activity was seen only in native-like DNA.

Antibodies

Erickson, Braun, Plescia, and Kwiatkowski (129) found that fractionated antisera to oligo- and polynucleotides inhibited linked markers equally in transformation but nonlinked markers unequally. DEAE-cellulose columns were saturated with either heat-denatured DNA or normal transforming DNA, and anti poly-dAT serum was passed through the columns. The majority of the inhibitory activity, about 90%, was removed upon passage through a column saturated with denatured DNA, but little inhibitory activity was removed by a column containing native double-stranded DNA. Prior incubation of antiserum and transforming DNA did not significantly alter the extent of inhibition, and exposure of antiserum to normal or sheared DNA did not affect its inhibitory activity. The length of pre-incubation with recipient

cells, however, did increase the extent of inhibition.

Effects of Chemical Agents on Transforming DNA

Mitomycin and Carzinophillin

Chemical action on transforming DNA bas been yet another aspect under study. Nakata, Nakata, and Sakamoto (130) reported that incubation of cells with mitomycin C (MC), a compound which breaks down bacterial DNA *in vivo* and accumulates masked deoxyribonucleosides, before DNA extraction impaired the transforming activity of isolated DNA more than 90%. However, MC treatment and removal from isolated DNA in no way impaired the transforming activity. Since MC apparently had no influence on DNA polymerase, deoxyribonucleotide kinase, or DNase activity, these workers suggested that it acted on DNA at the level of DNase activity. Okubo and Romig (131) showed transformation of MC-1, a mitomycin-sensitive mutant which can incorporate DNA to a DNase-insensitive state but in which only 10% of the donor marker can be re-extracted, to be impaired to a greater extent than the ability to incorporate DNA. Therefore, they suggested the mutation was in the cell's ability for recombination. Later work (132) indicated that neither MC nor carzinophillin (CF) led to base alteration or to single-strand breaks in the DNA. These workers hypothesized the inactivation of transforming DNA by CF through covalent interstrand cross-links. DNA inactivated by CF was relatively resistant to further heat inactivation at 100° for 5 minutes, and the inactivating effect of CF could be partially reversed by post-treatment incubation. Iyer and Szybalski's work (133) with MC, the polycyclic structure of which might favor intercalation into DNA prior to cross linkage,

showed that the cross-linked DNA denatured thermally but renatured spontaneously even with rapid chilling due to the maintenance of the original alignment through linkage. Cross-linking was viewed as the causative agent in the following: 1) interference with DNA replication; 2) rapid cell death; and 3) slight effect on the DNA's ability to transform for a given marker or to direct synthesis for one or a few proteins. These investigators suggested that such reversible cross-linking would permit turning on and off DNA synthesis and thus act in a regulatory manner. Experimentally only 10^{-4}% of the B. subtilis cells survived a 15 minute exposure to 12 µg/ml MC, and the transforming DNA isolated from these cells, which was estimated to have one cross link per molecular weight of 10 to 12 million, retained 20 to 30% of its biological activity. MC had no in vitro effect on purified DNA unless a cell extract was added. Terawaki and Greenberg (134) also showed preferential inhibition of bacterial DNA synthesis and progressive and extensive DNA breakdown with CF. Thermal denaturation curves indicated covalent cross linking between polynucleotide chains. Because in vivo post-treatment incubation gave greater partial restoration of the transformation activity at a faster rate than did in vitro post-treatment, an intracellular recovery mechanism such as excision of cross links was suggested. CF treatment of B. subtilis DNA in vitro reduced the transforming activity to 15% of the untreated control.

Nitrous Acid and Hydroxylamine

In work with nitrous acid and hydroxylamine (HA), which reacts specifically with cytosine bases, Freese and Strack (135) showed an

increase in mutagenic reactivity of DNA bases when the strands were partially or completely separated, as with increasing temperature or decreasing ionic strength.

Methyl-Methanesulfonate

Strauss and Wahl (136) found *in vitro* alkylation of DNA with methyl-methanesulfonate (MMS), a monofunctional alkylating agent which reacts primarily with guanine, to proceed as follows: alkylation, depurination, single-strand breaks, and chain scission. They attributed the low specific transforming activity of DNA alkylated *in vivo* to breaks caused by differential enzyme which *in vitro* acts preferentially on alkylated DNA. However, DNA containing alkyl groups was efficient in transformation, and therefore in replication. Strauss, Reiter, and Searashi (137) found a mechanism in *B. subtilis* for repair of damage caused by MMS. The recovery of a UV-sensitive strain in minimal medium plus thymine was understandable since the MMS damage was single-stranded breaks.

Polyfunctional Alkylating Agents and Radiomimetic Compounds

The radiomimetic agents azaserine, nitrofurazone, nitrosoguanidine, and proflavine and polyfunctional alkylating agents like nitrogen mustard and MC all share with UV light induced mutagenesis, cross-resistance, filament induction, and transformation inhibition abilities. This indicates a similar mechanism of action on DNA. However, only bifunctional alkylating agents and UV light can specifically inhibit DNA synthesis through cross link formation. Terawaki and Greenberg (138) found that azaserine inhibited the transforming factor of *B.*

subtilis both *in vivo* and *in vitro* but had no effect on *Hemophilus* transforming factor. Nitrofurazone and proflavine did not inactivate transforming factor *in vitro* which indicated either that they become activated in the cell, DNA replication is required for their activity, or they act indirectly with natural cell components first. A DNA replication requirement appeared to be the most plausible explanation. Therefore, these workers deduced that cross linking was not necessary in mutagenesis, filament induction, cross-resistance, or transformation inhibition. Strauss (139) indicated that alkylation and UV irradiation can sensitize DNA to further degradation without destroying its biological activity, perhaps by activating the DNA as a substrate rather than activating the DNase of the cell. UV-treated DNA was sensitive to enzymatic inactivation but not to temperatures of 50°. The transforming activity of alkylated DNA remaining after enzymatic inactivation was sensitive to 50°, and that remaining after heating to 50° was susceptible to enzymatic inactivation. Therefore, sites on the alkylated DNA may be originally susceptible to heat inactivation but resistant to enzymatic inactivation, and UV light may damage the DNA making it susceptible to enzymatic inactivation but not to heat.

Acriflavin

The inhibition of transformation by acriflavin was explained by Ayad (140) as an alteration in the donor DNA structure which prevents integration.

Oxidized Spermine

Early studies (141) showed that although spermine inhibited the growth of various bacteria, mostly Gram positive cocci, spermine oxidized by purified serum amine oxidase interfered with the growth of various bacteria and inhibited synthesis of some bacterial proteins. The carbonyls of oxidized spermine appeared responsible for the toxic effect since sodium borohydride, which reduces the dialdehyde to the alcohol, abolished toxicity. Since cations had no effect on the antibacterial action of oxidized spermine and the compound was active over a wide pH range a non-electrostatic linkage between the compound and the cell was postulated. Although protein synthesis was inhibited by oxidized spermine, nucleic acid synthesis continued, suggesting a possible interaction between oxidized spermine and nucleic acids as the cause of inhibition of protein synthesis. These workers hypothesized the reaction of the carbonyl groups with amino groups of purines and pyrimidines to form Schiff's bases. Inhibition by oxidized spermine increased with time and temperature of incubation. Further work on this compound by Bachrach and Eilon (142) indicated that the oxidized spermine-DNA complex was partially due to electrostatic forces since it only partially dissociated in 3 M NaCl and that the non-electrostatic bonds of the complex involved the two carbonyl groups. Oxidized spermine complexed to native DNA and behaved like a bifunctional alkylating agent which induces bridges between complementary DNA strands. The suspected cross links were confirmed by thermal denaturation curves, fractionation on hydroxyapatite columns, and sucrose gradient centrifugation runs. Oxidized spermidine, which has only one carbonyl group, was shown not to induce cross linking in DNA.

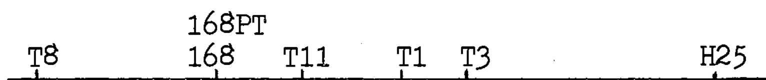
The effect of oxidized spermine on transforming DNA of *B. subtilis* was studied by Persky, Ephrati-Elizur, and Bachrach (143) who found marked reduction of biological activity. The interaction between oxidized spermine and DNA involved both ionic and non-electrostatic forces. The ionic forces were implicated by NaCl antagonism of the inhibition, and non-electrostatic factors by the thermal denaturation resistance of cross-linked DNA. In the latter respect, oxidized spermine resembled CF. However, oxidized spermine had a preferential inhibitory effect on transformation to histidine independence although linkage to tryptophan was not affected. Hence, oxidized spermine was assumed not to interfere with binding of DNA to the cell. This assumption was confirmed in studies where oxidized spermine did not affect the irreversible binding of ^{32}P-labeled DNA to competent cells. The higher sensitivity of the histidine region to oxidized spermine was explained on the basis of differing affinities of the compound for various purines and pyrimidines, such as a certain G-C distribution.

Linkage

Linkage of several markers on *B. subtilis* transforming DNA has been found, for instance, two histidine groups, one of which is closely linked to the indole marker (144). Nester and Lederberg (145) also confirmed the linkage of the $histidine_2$ and indole markers, but found these not linked to any of six others including methionine, methionine and lysine, glutamic, proline, valine and isoleucine, cystine, and streptomycin resistance. All genes controlling tryptophan synthesis which were studied by Anagnostopoulos and Crawford (146) appeared linked to each other and to a histidine marker. The markers

were arrayed in order of their biochemical sequence (Figure 7) (146) with only one inversion, and the histidine marker was mapped outside of the tryptophan cluster. The numbers in brackets in Figure 7 indicate the locations of various enzymatic blocks in mutants.

Bacteria blocked in [2] grow on indole or tryptophan and accumulate indole glycerol-3-phosphate; those blocked in [1a] grow on tryptophan and accumulate indole; those in [1b] grow on tryptophan and accumulate indole glycerol-3-phosphate; those in [3a] grow on indole or tryptophan and accumulate 1-deoxyribulose-5-phosphate; those in [3b] grow on indole or tryptophan and accumulate anthranilic acid; and those in [4] grow on anthranilic acid, indole or tryptophan. The genetic map of *B. subtilis* in this region appears below:



T3 represents the tryptophan$^-$ mutant blocked in [1b], 168 the indole$^-$ mutant blocked in [3a], 168PT the partial transformant of 168, T1 the indole$^-$ mutant blocked in [3b], T11 the indole$^-$ mutant also blocked in [3b], T8 the anthranilic acid$^-$ mutant blocked in [4], and H25 an unknown enzyme of the histidine pathway. Steps [3a] and [3b] constitute the inversion in the mapped section.

McDonald and Matney (147) found a trait for growth at 55°, as well as at 37°, closely linked to the streptomycin marker. McDonald (148) later attributed the inability of *B. subtilis* to grow above 51° to a mutation in the temperature sensitive (tms) region closely linked to the streptomycin region. The tms marker was consistently closer to the streptomycin marker than to the erythromycin marker by cotransfer index, and the sequential order was given as streptomycin, tms, erythromycin.

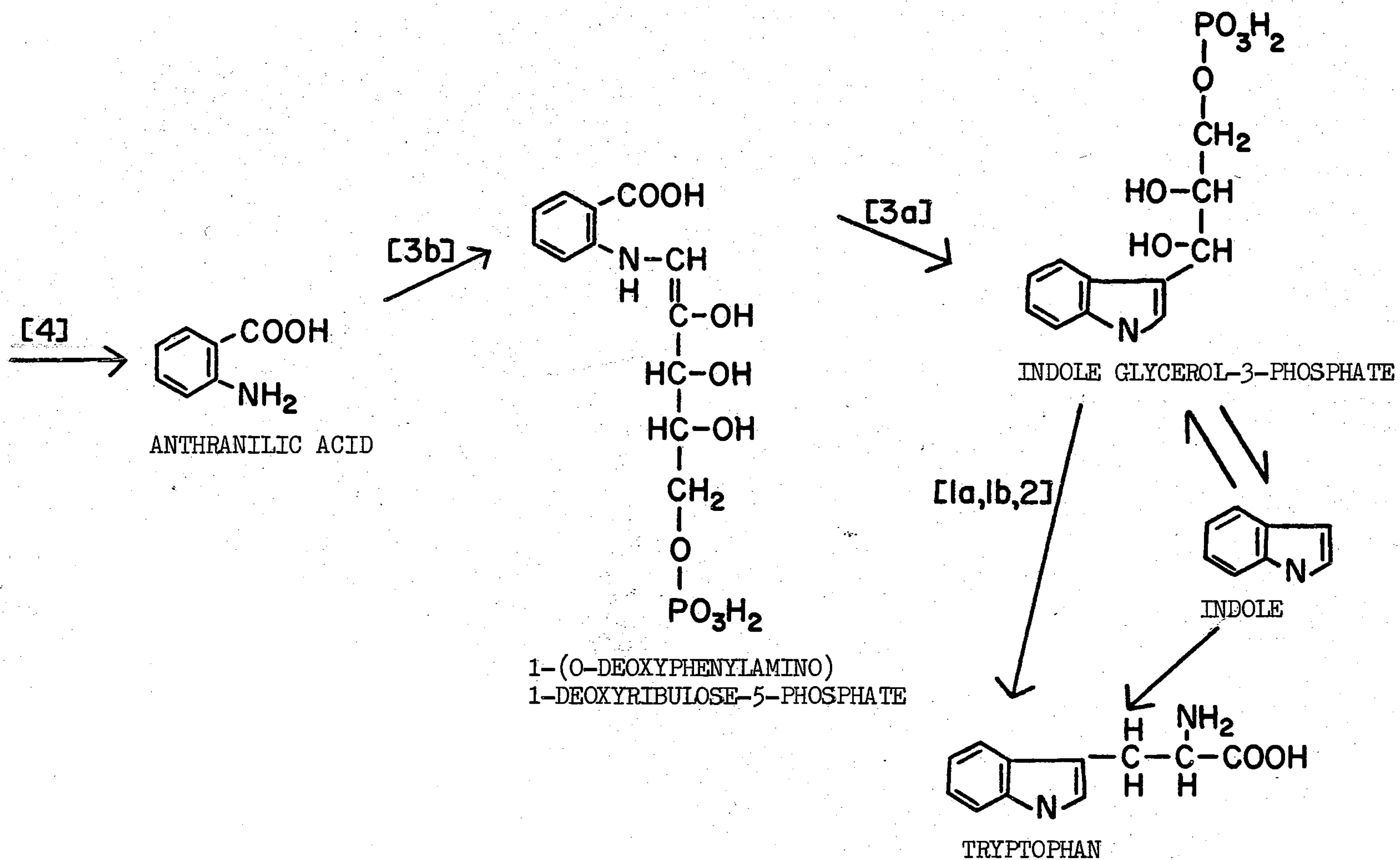


Figure 7. Tryptophan Biosynthetic Pathway (146)

Barat, Anagnostopoulos, and Schneider (149) noted that not all isoleucine and valine genes were clustered in B. subtilis, but instead appeared on two distinct DNA segments in transformation. One of the segments carried threonine deaminase and dihydroxy acid dehydrase linked to methionine, while the other bore reductoisomerase and one or more leucine markers with phenylalanine weakly linked. Mahler, Neumann, and Marmur (150) found three nutritional groups of arginine mutants which possessed linkage within each group but not between groups. One of the arginine markers was more heat resistant while the other two were not linked to an erythromycin marker which suggested transforming DNA segments were separate for the separate steps in arginine biosynthesis. Within the limits of his two-step transformation experiment, Polsinelli (151) found two closely linked mutations responsible for oxytetracycline (OT) resistance. These mutations possessed almost complete additivity at the phenotypic level. Bodmer (152) showed that DNase inactivated DNA carrying four linked markers most rapidly, followed by triplet, doublet, and singlet markers in order. Kelly (153) attributed the instability of linkage in transformation to the possibility of a high molecular weight DNA molecule reacting with two cells, one at each end of the molecule, thus breaking the linkage group. This explanation for linkage instability would, of course, depend on DNA concentration, cell concentration, competence, and the distance between markers.

In Vivo Transformation

Work by Takahashi (154) indicated that plating B. subtilis strains 168 (indole^{-}) and 170 (tryptophan^{-}, phenylalanine^{-}) together on

minimal media agar led to the formation of prototrophic colonies. These cultures were not competent at the time of plating, and heterologous DNA from Xanthomonas phaseoli or addition of DNase inhibited prototroph appearance. The occurrence of prototrophs apparently followed transformation by extracellular DNA although release of transforming DNA could not be demonstrated in cell-free filtrates of strains 168 and 170 even during competence development. Combinations of other auxotrophs showed similar results in plating on minimal agar. Therefore, B. subtilis transformation may occur on minimal agar without any special treatment. Strains which readily developed competence on minimal agar acted effectively as either donors or recipients, while strains which were poorly competent on minimal agar acted only as donors. More recently Ephrati-Elizur (155) has shown spontaneous release of transforming DNA during the exponential and stationary growth phases. This DNA is less fragmented, as measured by cotransfer indices, than that obtained by conventional isolation procedures. Borenstein and Ephrati-Elizur (156) also found germinating spores to release transforming DNA, the genetic markers of which appeared extracellularly in sequential order suggesting a correlation between DNA release and chromosome replication. DNA release may be dependent on prior chromosome replication, and the point of exit may be the membrane-chromosome attachment site. DNA release from synchronously replicating germinating spores has proved a useful tool in genetic mapping of B. subtilis. Transformation curves for different markers reach a plateau about the same time, but the DNA synthesis rate remains unchanged, indicating a possible physiological change to prevent further DNA release.

Zamenhof, Giovanni-Donnelly, and Heldenmuth (157) showed that a receptor strain of *B. subtilis* was transformed by DNA from mutant donors of various origins. In each case, the resulting transformant had the same mutation rate for the marker studied as did the donor. Since only DNA was transferred, the segment of DNA must have carried information determining its own mutation rate. Table V indicates the various types of transformable markers found in *B. subtilis* with references for each. Table VI summarizes the factors influencing transformation frequency.

TABLE V

TYPES OF TRANSFORMABLE MARKERS IN *B. subtilis*

Marker type	Reference
Amino acid	(17)
Antibiotic resistance	(87)
UV resistance	(39)
Motility	(212)
DNA or RNA base independence	(47)
Phage resistance or sensitivity	(158, 159)
Sucrase	(71)

TABLE VI

FACTORS INFLUENCING TRANSFORMATION FREQUENCY

Increasing	Reference	Decreasing	Reference
Absence of phosphate in minimal medium	(47)	Acridine orange	(47)
		Phleomycin	(47)
Mg^{++}	(47)	Puromycin	(167)
Yeast extract	(17)	5-Fluorodeoxyuridine	(167)
Protein removal from DNA	(17)	Complete RNA removal	(17)
β-Mercaptoethanol	(47)	Freezing and thawing of DNA preparation	(17)
		Oxidized spermine	(143)
		Antibodies to single-stranded DNA	(129)
		Proflavine	(138)
		Donor DNA treated with DNase	(17)
		Acriflavin	(140)

CHAPTER IV

THE STEPS IN TRANSFORMATION

The main steps comprising the transformation sequence are (160):

1. initial attachment of donor DNA to the competent cell,
2. entry of the DNA into the cell by a specific transport system,
3. synapsis of donor DNA and the resident DNA,
4. recombination between donor and resident DNA,
5. expression of the newly introduced genetic information.

Initial Attachment and DNA Uptake

DNA uptake involves two steps—reversible attachment of the donor DNA to the cell, followed by irreversible DNA transport to the cell's interior. Spizizen's work (71) showed cell walls synthesized under optimal growth conditions were capable of binding DNA irreversibly and preventing its penetration. Transformation was inhibited by heterologous DNA from unreleated organisms. Young and Spizizen (161) demonstrated that in competent cells irreversible DNA binding was influenced by temperature, hydrogen ion concentration, and aeration. The optimal temperature range for transformation was 34 to 37°, the pH optimum was 6.9 to 7.4, and aeration was necessary for optimal irreversible uptake of DNA and transformation. Divalent cations such as Ba^{++}, Sr^{++}, Ca^{++}, and Mg^{++} were required, and their postulated role in the stabilization of DNA was based on the following evidence:

1) ions stimulating or inhibiting DNA incorporation are similar generally to those stabilizing or destabilizing DNA; 2) heated DNA is less readily incorporated in Pneumococcus and B. subtilis transformation systems but still retains its biological activity; 3) critical molecular size is necessary for incorporation since Mg^{++} and Co^{++} are bound almost stoichiometrically but only the former permits transformation in B. subtilis. Under suboptimal conditions or with metabolic inhibitors, transformation was decreased more than was DNA incorporation. Incorporation of ^{32}P from labeled DNA increased as phosphate was depleted, perhaps due to phosphate incorporation after the cleavage of a terminal DNA phosphate.

Saito and Kohiyama (162) reported B. subtilis transformants proportional to the DNA concentration up to the saturation point. Addition of both homologous DNA and polyphosphate prior to transforming DNA shortened the lag period in transformant appearance and stimulated transformation. The inducing effect shown by homologous DNA was not sensitive to DNase. The use of ^{32}P-labeled DNA also indicated immediate adsorption of polyphosphate after DNA addition. In summary, these findings indicated the induction of a DNA-permeating system. However, Young and Spizizen (40) showed that DNA uptake could be correlated with transformation only under carefully controlled conditions.

Levine and Strauss (163) showed DNA uptake in B. subtilis occurred with immediate adsorption of DNA to the cell surface followed by subsequent irreversible uptake. DNA which has penetrated the cell wall or is attached to the cell and is insensitive to DNase is considered taken up irreversibly. A lag in the appearance of transformants was evident only when DNase was used to terminate transformation, not when

cells were washed to remove unbound DNA. DNA uptake has an energy of activation of 13.9 kcal (163) and may be inhibited by oxidative phosphorylation inhibitors such as cyanide and 2,4-dinitrophenol (40).

DNA penetration into the cell has been studied through the effect of DNase treatment on the appearance of transformants. A lag period of 2 to 4 minutes, thought to be involved in DNA uptake, was noted in transformant appearance of B. subtilis exposed to DNA for various times and was presumed to be the time required to synthesize an inducible enzyme. Strauss (164, 165) confirmed the previously suspected lengthwise entry of DNA into the cell and showed the rate of uptake to be about 55 nucleotide pairs per second. However, only part of the lag period was involved in DNA transport.

Kammen, Wojnar and Canellakis (52) suggested that transforming DNA was compartmentalized after cellular entry, perhaps within the cytoplasmic membrane, while excess DNA outside the membrane was freely exchangeable with DNA in solution as long as competence persisted. Intracellular competition of transforming DNA with homologous or heterologous DNA was also postulated. Young (64) reported newly transported DNA may bind to the cytoplasmic membrane and bound DNA was most often associated with structures resembling fore-spores. Tritium-labeled DNA was shown adjacent to the cell wall after uptake. Hirokawa and Ikeda (166) showed B. subtilis protoplasts capable of greater DNA uptake and greater yield of re-extracted DNA than intact cells. They noted the formation of recombinants of exogenous and endogenous DNA and of two exogenous DNA segments but observed no DNA synthesis. Therefore, the breakage-reunion hypothesis for recombination was most applicable.

Kammen, Beloff and Canellakis (167) reported the stabilization of transformants by amino acids, and suggested that after cellular entry, DNA stabilization requires a polypeptide. The addition of amino acids reversed the inhibitory effect on transformation of puromycin and 5-fluorodeoxyuridine and increased resistance to actinomycin D. Although potential transformants were not growing, at least two biosynthetic functions were operative immediately following DNA uptake. One function was protein and/or RNA synthesis, and the other was polypeptide synthesis not mediated by m-RNA. McCarthy and Nester (168) have suggested that a heat-labile factor is required for DNA uptake and/or retention. Once this factor is inactivated, the cell does not repair or replace it for at least several hours. The general biosynthetic latency of the competent cell probably prevents immediate replacement of the factor.

Recently Erickson, Young and Braun (169) showed transformation inhibition by antibodies to single-stranded DNA at a time when the reaction is DNase insensitive. Both the rabbit gamma globulin and the DNA appeared to bind to the plasma membrane or perhaps to some other lysozyme-resistant portion of the wall. Gamma globulin specific for single-stranded DNA did not interfere with the initial interaction between cell and DNA, but did affect the later DNase-insensitive cell-associated DNA. These workers suggested a complexing of DNA to the cell surface and conversion to single-stranded DNA prior to transport across the membrane.

Synapsis

Following DNA uptake, synapsis between the donor DNA and the cell

genome occurs. Hydrogen bonding may be involved in the recognition by the donor DNA of its complementary area on the cell genome (111).

Integration of Recombination

Synapsis of these two DNA segments is followed by integration during which time the recipient cell gains the information encoded on the donor DNA. The two possible mechanisms by which a cell may acquire new heritable traits are: 1) the message could be transmitted without physical transfer by the copy choice scheme, or 2) the exogenous material could be physically inserted into the recipient genome by the breakage-reunion method (170). Investigations were begun to prove one or the other of these mechanisms was operative in _B. subtilis_ recombination. Using ^{32}P-labeled DNA, Young and Spizizen (40) noted that specific DNA incorporation was dependent on the DNA preparation as well as on physiological factors and was represented by the following equation:

$$\text{specific incorporation} = \frac{\text{DNA incorporated}}{\text{No. of cells transformed}}$$

Bodmer and Ganesan (171) isolated DNA from a multiply marked _B. subtilis_ grown in $^{15}N^{2}H$ medium with added ^{3}H-thymidine. Competent cells were prepared in $^{14}N^{1}H$ medium with ^{32}P, and transformation was terminated at 10 to 30 minute intervals with DNase. Donor and recipient DNA's differed in buoyant density and were traced by differential ^{32}P and ^{3}H counting. Donor atoms appeared in native donor, in native recipient, in hybrid, and denatured donor DNA. The latter was biologically inactive, but the others contained both donor and recombinant genotypes. The relative numbers of donor atoms found in the heavy strata decreased with growth after transformation. Additionally,

denaturation by heat, alkali, or shearing increased density separation of ^{3}H and ^{32}P counts but showed no predominantly donor density material. These data provided evidence that portions of intact donor DNA were built into the recipient genome and held there by bonds resistant to shearing or denaturation. However, these experiments could not clearly distinguish between double-stranded and single-stranded integration. Single-strand integration was indicated, and assuming this to be true, an integrated region would consist of about 8.4×10^3 nucleotide pairs (171).

Lorkiewicz, Opara-Kubinska, and Szybalski (172) labeled protein- and RNA-free DNA preparations of donor DNA with ^{32}P and recipient cell DNA with 5-BUdR, mixed and centrifuged them in CsCl. Since good separation was obtained with just physical mixing of the two DNA types, ^{32}P-labeled DNA was added to competent BUdR-labeled cells, followed by DNase and washing. Centrifugation of the re-extracted DNA showed a definite shift in the recipient DNA to a heavier density indicating actual insertion of the donor DNA into the genome. Venema, Pritchard, and Venema-Schroder (173) demonstrated an eclipse period in _B. subtilis_ shortly after DNA uptake similar to that of other bacteria. Such an eclipse period might result from denaturation of donor DNA after uptake. Recovery of activity after the eclipse phase was separate from integration, and only one strand of the transforming DNA duplex seemed to be integrated into the recipient genome. Vestri, Felicetti and Lostia (174) reported transformation by hybrid DNA of _B. subtilis_ occurring through one strand since information from only one of the hybrid strands was later observed in the recipient cells. However, using _B. subtilis_ DNA with histidine^{+} on one annealed strand

and tryptophan$^+$ on the other strand, Bresler, Kreneva, Kushev, and Mosevitskii (175) showed transformation of a double auxotroph to a prototroph, thereby indicating that both DNA strands act as templates for m-RNA. They proposed the following methods by which this could occur: 1) both genes may be read in opposite directions to make DNA active for these enzymes; 2) only one strand of the m-RNA can act as a protein synthesis template while the other must be copied a second time in order to act as a template; 3) there may be a third polyribonucleotide strand complementary to both DNA strands.

An addition controversy arose when Bazill (176) suggested integration of both strands of the transforming duplex based on the expected and observed segregation lag in a culture. Figure 8 (176) shows that a marker on only one strand leads to a constant number of such cells in the first division, and the lag then disappears in the second division. But if markers are on both strands, there is no lag predicted. Several difficulties faced this hypothesis, for instance, not all cells are uninucleate, not all have only one marker gene per nucleus, and the biosynthetic latency of competent cells makes determination of the onset of segregation uncertain. However, Bazill solved the latter problem by using penicillin to discern the beginning of cell division after amino acid starvation and addition. Experimentally a segregation lag of not longer than one generation was seen, while that expected for integration of markers on one strand was 2.36 generations and for markers on both strands 1.36 generations. Pene and Romig (177) found a fraction of donor DNA physically associated with recipient DNA after uptake and suggested the following explanations: 1) a failure to separate macromolecular aggregates of donor

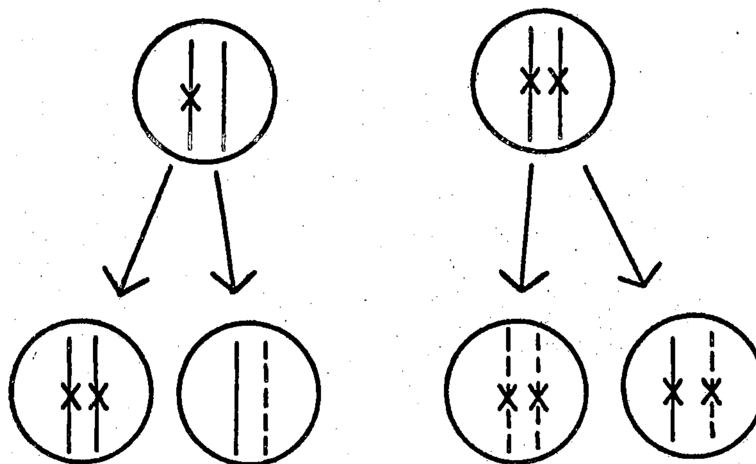


Figure 8. Segregation Lag in Single or Doubly Marked DNA (176)

and recipient DNA by CsCl centrifugation; 2) degradation of donor DNA by metabolic processes; 3) donor DNA became single-stranded after entry; 4) the material contribution from exogenotic DNA is a result of genetic recombination. Since these workers found no single-stranded donor DNA and no non-specific aggregation of donor and recipient, they assumed the latter suggestion to be true. Chilton (178) showed that 10^{-3} M EDTA inhibited the transforming activity of both native and residual DNA about three fold while separated complementary strands of DNA became the most active component, suggesting that either strand of duplex DNA may act as the donor in transformation. Becker (179) reported that transformation was achieved by integration of either single- or double-stranded segments of DNA. Enzymatic removal of cross links in double-stranded DNA must occur during the first 30 minutes of the process, or exclusion effects will prevent integration of DNA in the vicinity of cross links. Ayad and Barker (180) recently found that the intermediate density hybrid of donor and recipient DNA possessed biological activity of both donor and recipient markers. Since denaturation resulted in only one component, these workers suggested integration had occurred in both strands of the recipient DNA.

Venema, Pritchard, and Venema-Schroder (181) showed abnormal physical properties in DNA extracted from competent cells 7.5 minutes after DNA fixation. These abnormalities, which included resistance to thermal denaturation and reduced renaturation capacity, indicated incomplete integration. The following three stages of integration were postulated: 1) recovery from the eclipse phase, a phenomenon perhaps due to donor-recipient DNA complexing; 2) development of

recombinant-type activity and resistance to denaturation and renaturation; 3) development of recombinant-type activity with normal physical properties. Wingfield, Kerr, and Durham (182) grew B. subtilis for short periods in the presence of proflavine or actinomycin D and showed chain production which might invalidate the plating count of transformants. They suggested that absorbancy measurements be used to score transformation more accurately when chaining is suspected or confirmed. Since proflavine and actinomycin D bind to nucleic acids, their effect on transformation 60 minutes after DNA addition was also studied. Even after 60 minutes, integration was not complete and could be inhibited. Inhibition of DNA-directed RNA synthesis was considered, and the possibility of an enzyme system involved in the inhibition was suggested (182).

The physical association found between donor DNA and resident genome in several studies (171, 177, 180) indicated the occurrence of integration in B. subtilis through breakage and reunion. Additionally, actinomycin, which binds DNA, prevented breakage of the exogenous DNA and formation of recombinants (166). Hirokawa and Ikeda (166) showed recombination in the absence of protein synthesis, an energy supply, or a normal level of DNA synthesis. Finally, Bodmer (183) has proposed a model whereby donor DNA is integrated at the stationary replicating point of the competent cell which is in a state of suspended DNA synthesis. This replicating point was also associated with the cell membrane, a location where it would be readily accessible for contact with donor DNA. Other work (49) has shown donor DNA predominantly integrated into the old strands of the recipient DNA. The lower integration efficiency of DNase-treated DNA was attributed to

single-strand breaks which inhibited the unwinding process involved in mediation of synapsis by interchange of base-pairing partners. Integration of a strand containing a break may be lethal through inhibition of subsequent replication. In addition, integration of small DNA pieces may interfere with the repair process and leave lethal gaps. Laird, Wang, and Bodmer (184) grew cells in heavy $^{15}N^{2}H$ medium containing 2-^{14}C thymidine and transferred them midway through the competence regimen to light $^{14}N^{1}H$ medium. Transforming DNA was added to competent cells, and DNA was then extracted. This DNA showed donor and recombinant transforming activity predominantly in the hybrid region while the remainder was in the fully replicated light region. Recombinant DNA was entirely hybrid in density after recentrifugation. The method of cell lysis used preserved the structure of the replication points, however, integration did not occur preferentially at these points. This study did not distinguish whether new replication points were induced by recombination or extensive DNA repair synthesis occurred after integration. And the existing replication points, if any, may have been especially sensitive to the DNA isolation procedures.

Erickson and Braun (185) demonstrated a cyclic shift in the relative frequencies of transformation of non-linked markers by adding DNA at different times during the 3 hour period of general competence of a partially synchronous culture. Transfer of a spore culture to fresh medium induced synchrony. DNA replication of recipient cells was also synchronized. The hypothesized DNA-membrane attachment, the slow decay of donor DNA at this site, and integration at the time of replication reportedly accounted for the cyclic

changes (185). Double transformants were frequent when both markers were proximal to the assumed replication point at the time of DNA addition, but infrequent when only one of the two markers had already passed the replication point. DNA addition to a competent culture at various times allowed for approximate mapping of the markers under study. Ganesan and Lederberg (186) reported that the *B. subtilis* genome consisted of 1.8×10^6 nucleotide pairs and that the site of attachment of the replicator was protein. That the cell wall-membrane complex was the site of DNA synthesis was shown when, after cell lysis, the pellet consisting of cell wall fragments incorporated all four deoxynucleoside triphosphates into DNA using DNA primers. The synthesis was dependent on Mg^{++} and required all four triphosphates. Synder and Young (187) later showed that the origin and terminus of the *B. subtilis* chromosome are associated with the cell wall membrane and protected from nuclease action. They also indicated that the site of membrane-chromosome attachment may not vary during growth. However, recent work by Archer and Landman (188), in which the chromosome of a tryptophan$^-$, thymine$^-$ double auxotroph was aligned at the terminus by amino acid starvation, indicated the apparently free cellular entry of transforming DNA and the formation of transformants for markers both near and distant from the terminus. These workers felt that the hypothesis of marker entry being dependent upon DNA homology at the replication fork could be disregarded, but that DNA might remain inside the cell during movement of the replication fork and become integrated at a later time.

Howard-Flanders and Boyce (189) suggested the following four steps in DNA repair: 1) single-strands of double-stranded DNA are

interrupted in the following ways: (a) radiation-induced chain breakage, (b) enzymatic excision of damaged bases, or (c) recombination enzymes acting during early stages of genetic recombination; 2) nucleotides are released presumably through the action of enzymes on free single-strand ends, but only for a limited distance in normal cells; 3) DNA twin helix is reconstructed by DNA repair polymerase adding to single-strand ends with the single strands serving as initiators and intact strands as templates; 4) joining enzyme completes the phosphodiester backbone. DNA repair occurs after treatment with some mutagenic agents and during genetic recombination such as transformation. The discovery of _E. coli_ K-12 mutants which conjugated and accepted genetic material normally from suitable donors but appeared defective in recombination and were abnormally sensitive to UV light suggested common steps between DNA repair and recombination mechanisms (189). The levels of endonuclease I, exonuclease I, II, and III, and DNA polymerase in recombination$^-$ mutants were no different from those of the original strain which indicated a defect in control or regulatory function rather than a defect in the recombination enzymes. The common steps in repair and recombination may involve controlled widening of single-strand gaps, DNA repair synthesis, and rejoining of phosphodiester bonds.

Replication

The next step in transformation is chromosome replication. Yoshikawa and Sueoka (190) showed definitely oriented replication of the _B. subtilis_ chromosome and suggested the following two modified models: 1) subunit model in which the subunits replicate more or

less independently with polarity within each subunit; 2) replication starts from both ends of a subunit or chromosome. This work also indicated that chromosomes in the stationary phase cell were in completed form. Yoshikawa and Sueoka (191) tested the validity of the former experiment by moving cells from the isotopic medium in which they were grown to a light, unlabeled medium. Oriented replication was again noted, and transfers during the stationary phase exhibited more synchrony than did those during exponential phase when synchrony was only partial and the resting period was longer. Yoshikawa, O'Sullivan, and Sueoka (192) found no evidence of chromosome polarity in _B. subtilis_ W168 when marker frequencies of DNA from the exponential phase cells were compared to those of stationary phase cells. This exception to previous experimental evidence was, however, due to a less rigid regulation of chromosome replication, not to a different replication mechanism. DNA from spores possessed polarity indicating that completed chromosomes do not accumulate in the stationary phase cells of strain W168. Multi-fork replication appeared in rapidly growing cells and led to the conclusion that repression of reinitiation during replication may be broken.

Rolfe (193) found that CsCl centrifugation of DNA samples isolated from exponentially growing cultures of _E. coli_, _B. subtilis_, and _B. megaterium_ showed heavy satellite bands. The work on _E. coli_ indicated that DNA may enter a state when it is structurally different from native DNA. The heavy band was suggestive of a collapsed structure in which the conserved subunits were not dissociated and which might serve as a template for replication more readily than native DNA. The light component seen upon centrifugation was unstable,

subject to low recovery from density gradient experiments, and was possibly associated with specific proteins such as polymerases or nucleases.

Oishi and Yoshikawa (194) studied synchronous and dichotomous replication of the B. subtilis chromosome during spore germination. Spores contained completed chromosomes with a sequential order of replication from origin to terminus as in vegetative cells. Dichotomous replication was observed in enriched medium just as in the exponential growth phase. Schemes for normal and dichotomous replication appear in Figure 9 (194). The following are advantages of dichotomous replication: 1) generation time is reduced; 2) initiation is not dependent on completion of replication; 3) it is a well regulated system since reinitiation is repressed until the first replicating point reaches approximately mid-chromosome; 4) it results in the best synchrony.

Okazaki, Okazaki, Sakabe, Sugimoto, and Sugino (195) postulated another method of replication in B. subtilis in which the daughter strands at the growing point may look like ______ _____ ____ ___ __ _ "units" which are joined by phosphodiester bonds at nonterminal positions. The possibility of artificial breaks occurring in the new strand during selection was considered unlikely. This hypothesis of discontinuous replication is illustrated in Figure 10 (195).

More recently in vivo studies of DNA replication by Oishi (196) indicated that single-stranded DNA was isolated from B. subtilis and E. coli as the initial product of DNA synthesis in cells which were pulse-labeled with tritiated thymidine. This first intermediate was transient and rapidly converted in vivo to a double-stranded form.

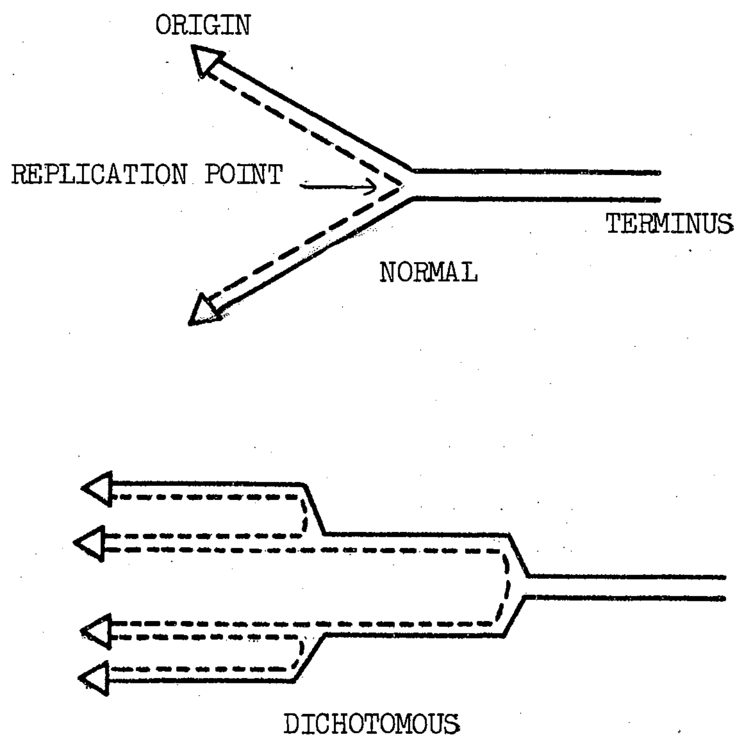


Figure 9. Normal and Dichotomous Replication (194)

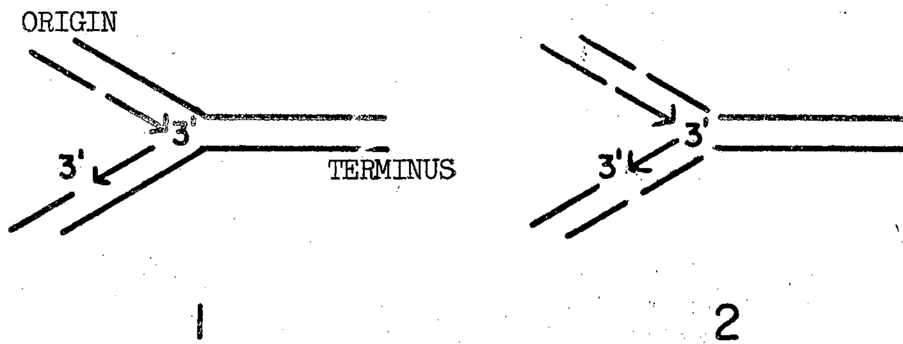


Figure 10. Discontinuous Replication (195)

Oishi (196) suggested the following three replication models:

1. The single-stranded form is the initial product and the template copying is mediated by some protein, perhaps polymerase. The single strand then forms native DNA in combination with the template.
2. Prereplicative single-stranded template is twisted or stretched, preventing alignment of new DNA in a normal double-stranded form. Stacking forces are reduced or absent in this case. Isolation procedures may release this single-stranded form.
3. Template copying occurs on double-stranded DNA, forming a transient triple-stranded form. Polymerase must be able to recognize the bases inside the double helix.

Oishi (197) later reported that the first intermediate was a DNA polymerase product of low molecular weight, perhaps single-stranded or loosely bound to the template. The first intermediate was converted to a double-stranded second stage which contained single-stranded segments and gaps in the newly synthesized strand. Single-stranded segments were not shown to exist _in vivo_, and ligase was suggested as a means for patching the gaps.

DNA Polymerase

B. subtilis was a more desirable source for isolation of DNA polymerase, which is found in association with the cell wall-membrane complex, because it possessed low levels of exonuclease activity and DNA phosphatase-exonuclease activity and lacked endonuclease activity (198). The DNA template determined nucleotide sequence through base

pairing. Falaschi and Kornberg (199) showed equivalent polymerase levels in B. subtilis spores and vegetative cells. However, polymerase within the spores was far more heat stable than in vegetative cells. Further work on partially purified B. subtilis polymerase indicated the presence of nuclease and ligase activity and the ability for limited synthesis of biologically active DNA without a template through semiconservative replication (200). Ligase increased the biological activity of both template and product equally, but the addition of ligase during polymerase action inhibited synthesis more than four fold, presumably due to endonuclease activity.

Englund, Deutscher, Jovin, Kelly, Cozzarelli, and Kornberg (201) reported the following properties of polymerase:

1. polymerase is apparently a single polypeptide chain of 109,000 molecular weight containing one sulfhydryl group and one disulfide group,
2. one binding site for four deoxynucleoside triphosphates,
3. one binding site for the DNA template,
4. polymerase binds to helical DNA only at nicks or ends and to multiple sites on single-stranded DNA,
5. polymerase catalyzes polymerization, PP_i exchange, and degradative reactions of pyrophosphorolysis and hydrolysis of all which occur at the 3' end of the DNA with the exception of hydrolysis which occurs at both the 5' and 3' ends.

In his review Kornberg (202) suggested that the sulfhydryl group was not in the active site of polymerase. The following five major sites were named in the active center: 1) a site for a portion of the DNA

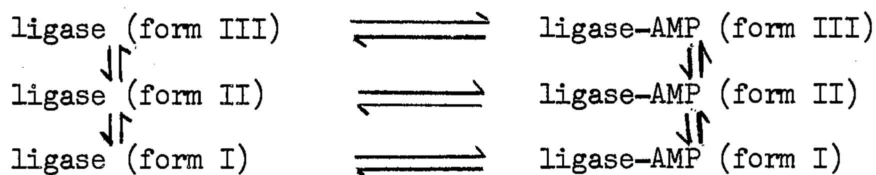
template; 2) site for primer with opposite polarity to that of template; 3) site for special recognition of 3'-OH end of the primer; 4) a triphosphate site; 5) a 5' phosphate hydrolytic site.

Polynucleotide Ligase

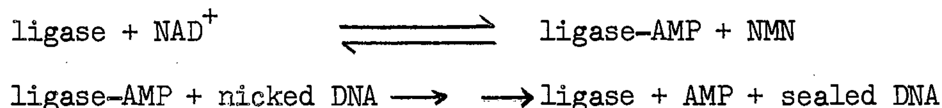
After DNA polymerase has replicated the chromosome or repaired the chromosomal damage to the point that only a single phosphodiester linkage is lacking for completion, ligase catalyzes the formation of the last phosphodiester bond. Polynucleotide ligase, first discovered in 1967, was isolated from *E. coli* (203) and required the following (203): 1) polynucleotides to be joined must be part of a double-stranded structure; 2) either Ca^{++} or Mg^{++}; 3) a heat stable cofactor later shown to be NAD^+. Recent work on more highly purified *E. coli* ligase suggested the following mechanism which included conservation of the energy released from the PP bond of NAD^+ within the new PP bond linking AMP to the 5' end of DNA (204):

1. NAD^+ + ligase ⟶ enzyme-AMP + NMN
2. enzyme-AMP + DNA ⟶ DNA----------5'phosphoryl-P-adenylate
3. DNA-----5'phosphoryl-P-adenylate ⟶ 5'------3'OH + AMP

Other work showed complexing between a purified fraction of *E. coli* ligase and NAD^+ in the absence of DNA (205). This complex, form III, was interconvertible with two new chromatographically distinguishable forms, I and II. Form I was inert with DNA and appeared to be a subunit with half the molecular weight of form III. Form II interacted with DNA possessing single-stranded breaks but only at much higher concentrations than the original. All three forms must interconvert in the following manner:



In two independent investigations Olivera, Hall, and Lehman (206) and Hall and Lehman (207) proposed the following mechanism for ligase action:



Replication Mutants

Mendelson and Gross (208) reported the isolation of a temperature-sensitive mutant of *B. subtilis* in which RNA and protein synthesis continued at 45° but DNA synthesis soon decreased greatly. This mutant apparently allowed completion of the replication which was under way but prohibited reinitiation at 45°. The mutant selection procedure involved more rapid killing of strain 168ts$^+$ (indole$^-$, thymine$^-$) by incubation with BU rather than lack of thymine. Cells were grown in enriched minimal medium at 30° over night, washed free of thymine, and suspended in the presence of BU. After incubation, BU was removed and the cells were suspended in minimal medium plus thymine for over-night growth. Samples were then plated on yeast tryptone agar, replicated on yeast tryptone agar, and grown for 6 hours at 45°. Colonies appearing on the master plant but not on the 45° plate were picked for more testing.

Subsequently, five classes of temperature-sensitive mutants were found in *B. subtilis* (209). Two mutant classes were defective in initiation of replication but were able to complete the replication

cycle in progress at the time of transfer to high temperatures. Two more classes were defective at some step in DNA synthesis, and the fifth class had a high rate of mutation for all characters tested in the temperature range where DNA synthesis occurs. Copeland and Marmur (210) also isolated temperature-sensitive mutants of B. subtilis Mu8u5u16 which were characterized at 34° and 48° by growth curves, viability, morphology, DNA synthetic ability, RNA and protein synthesis using the incorporation of tritiated precursors. Three of these mutants were blocked in DNA synthesis at 48° while RNA and protein synthesis continued for at least 60 minutes. A fourth mutant showed reduced incorporation of tritiated thymine at 48° for about 20 minutes before it ceased completely. After one doubling time, thymine incorporation began again. Mutant T47 acted similarly to the fourth mutant, but did not resume thymine incorporation. Another mutant behaved like the parental strain, that is, incorporated tritiated thymine at an enhanced rate but stopped before the end of one doubling time. All these mutations acted directly or indirectly on DNA synthesis. Tritiated thymidine uptake and/or DNA synthesis was the earliest measured trait found to be affected in these mutants.

Expression

Following recombination of the donor and recipient DNA's and replication of the new transformant's genome, expression of the recently acquired traits may be seen phenotypically. In an attempt to distinguish between the transformation process and its subsequent phenotypic expression Jensen and Haas (211) found that transient supplementation of solid medium with nutritional factors during incuba-

tions of up to 5 hours had no effect on the subsequent transformation frequency. However, the transformation frequency increased linearly with incubation on complete medium. Effective separation of transformation and expression was also shown. Stocker (212) discovered a lag in the expression of motility and tryptophan synthesis for 3 hours following transformation and suggested the biosynthetic latency of competent cells as an explanation. Micromanipulation of each transformant type showed the majority to produce only transformed progeny, a mixture of transformed and non-transformed progeny, or a mixture of two kinds of transformed progeny. Some types yielded only untransformed progeny or progeny transformed only at a locus linked to those concerned with motility or try synthesis. Partial heterozygotes were found up to ten generations after DNA uptake. In nonmotile clones derived from motile clones Stocker (212) found the unilinear transmission of motility to one in four descendants. This phenomenon he attributed to the persistence of corresponding numbers of units of some product of the unincorporated flagella^{+} gene, perhaps either a flagella or a cell wall fragment carrying several flagella.

Joys and King (213) demonstrated that transformed cells passed through a stage in which they were unable to manifest transformation if exposed to physical forces on the surface of solid medium. This is perhaps correlated to the eclipse phase mentioned previously. Incubation in liquid medium before plating eliminated the susceptibility to surface forces. Green and Colarusso (214) showed the α-amylases of two B. subtilis strains to differ in electrophoretic mobility, thermal sensitivity, K_M, and repressibility. Transformation of the strain possessing repressible activity by the non-repressible one

indicated the following: 1) one-step transformation; 2) nonrepressibility transferred simultaneously; 3) K_M and thermal sensitivity of acquired amylase were the same as those of the donor but the electrophoretic pattern showed two new isozymes. Acquisition of the donor-type enzyme resulted in the complete repression of recipient amylase enzymes. However, when loss of the transformed amylase occurred in transformants, repression of the original recipient amylases was eliminated. These findings indicated nonallelism of the two amylase types.

In addition to transformation, several reports of transfection in _B. subtilis_ have been documented. DNA isolated from SP10, ϕ1, ϕ25, ϕ29, SP82, SP3, and SP01 transfect _B. subtilis_ cells. In the transfection process recipient cells must be competent as in transformation, and often more than one phase DNA molecule must be taken up for production of complete phase particles. The DNA binding and uptake steps are thought to be common to both transformation and transfection. However, specific competence studies in transformation are hampered by required recombination and expression of the transforming DNA before transformants can be recognized. Bott and Wilson (39) demonstrated the competence peak for both transformation and transfection 3 hours after cessation of logarithmic growth, and confirmed the association between competence and a specific growth stage. The following were listed as advantages of transfection: 1) the entire ϕ29 genome is small, approximately the size of an average isolated bacterial DNA segment, and there is less chance of shearing; 2) infectivity is linear with DNA concentration; 3) integration may not be necessary for expression, a condition not yet verified. Trans-

fection was inhibited by glutamic acid and alanine, which is understandable since there has been no reported transformation of alanine$^-$ or glutamic acid$^-$ Bacilli. Both alanine and glutamic acid are major components of the mucopeptide, and their absence from the medium could allow wall expansion and binding of DNA to the membrane.

Although the binding of and uptake of DNA may be common to both transformation and transfection, recent work by Dubnau, Davidoff-Abelson, and Smith (215) indicated different pathways for recombination in transformation and transduction. Pathway I, which is operative in transformation, allowed much looser linkage, was more tolerant of foreign DNA, and usually handled lower molecular weight DNA segments. The base sequence inhomology was most likely the property of foreign DNA recognized in transduction. The pathways could be distinguished by rec-1, a mutant defective in transformation. PBS-1 transduction between B. subtilis W23 and 168 was of much lower frequency than transduction between homologous donors and recipients. However, transformation frequencies were similar with either homologous or heterologous donors and recipients indicating transfer of smaller DNA segments in transformation.

SUMMARY

Development of competence, the initial phase and necessary component in transformation, is under genetic control and is correlated with a specific growth stage. Protein synthesis and chelation of cupric ions are required although most of the culture is not actively growing and the rate of DNA synthesis may be very low. Competence development is inhibited by excessive amounts of cell wall precursors. The cell wall composition of competent and noncompetent cells differs—competent cell walls contain more galactosamine and alanine while noncompetent walls have more glutamic acid, diaminopimelic acid, and glucosamine. An autolytic enzyme is involved in competence development, and an extractable competence factor, apparently a protein, is found in competent cells. Studies directed primarily at competence must also involve the later steps of transformation since transformation and, therefore, competence are seen only upon completion of the entire sequence. From these undisputed bits of knowledge, competence seems to be a property of the cell wall-membrane complex, perhaps the result of DNA penetrating gaps left by the autolytic enzyme.

The transformation sequence (Figure 11) includes the following: 1) initial attachment of DNA to the recipient cell; 2) uptake of DNA; 3) synapsis of donor and recipient genome; 4) integration or recombination; 5) expression of the new genotype. Initial attachment is immediate, and uptake is influenced by temperature, pH, aeration, and divalent ions. After uptake, donor DNA is adjacent to the cell wall.

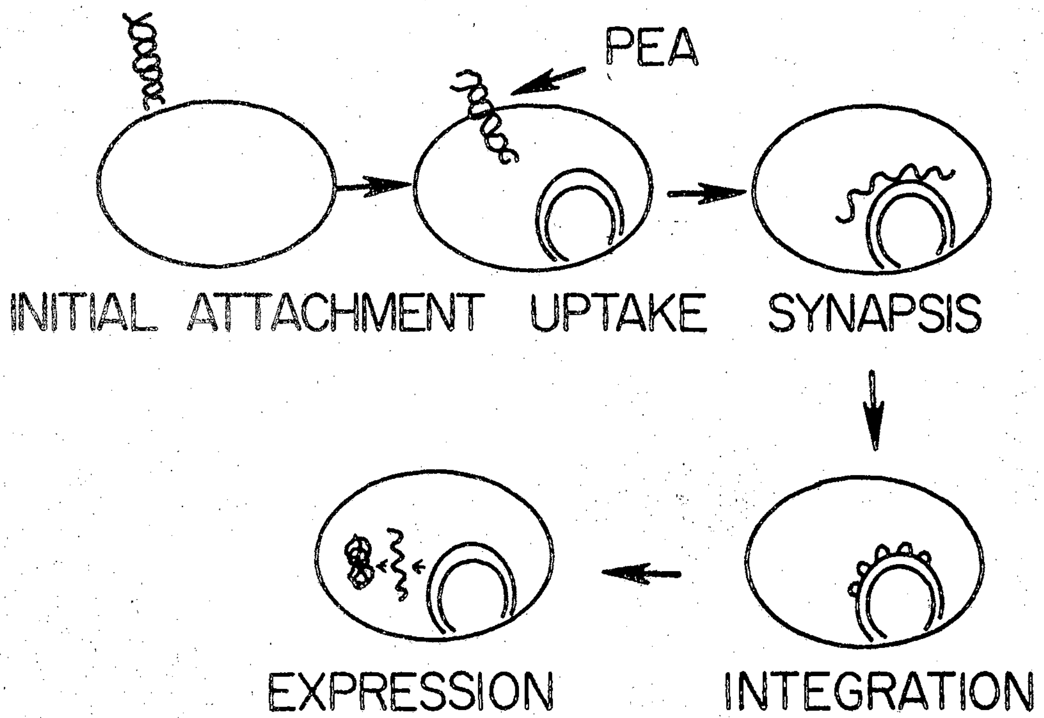


Figure 11. Transformation Scheme

The entering DNA may be single-stranded. The recognition of complementary areas of DNA in synapsis apparently involves hydrogen bonding. Integration occurs via the breakage-reunion method, but a controversy still exists over integration of single-stranded or double-stranded DNA. Following integration of donor DNA, the chromosome is replicated via a transient single-stranded DNA which is converted to the native form by association with its template. DNA polymerase and ligase are involved in both integration and replication. Expression of the new genotype is separate from the earlier transformation steps and may be influenced by physical forces on the surface of solid medium.

As with competence, study of individual steps in transformation is hampered by the dependence of each upon completion of the entire process. If experiments showing integration of both strands are correct, uptake may involve both double- and single-stranded DNA. The transformation frequencies reported vary greatly. Some workers cite routine frequencies in the range of 1.0% while others feel justified in reporting results with only 0.03% transformation. A more limited range of variation in published results would be desirable.

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