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Studies on the transformation of *Escherichia coli* K-12

Liveris, Dionysios, Ph.D.

City University of New York, 1988

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STUDIES ON THE TRANSFORMATION OF *Escherichia coli* K-12

by

DIONYSIOS LIVERIS

**A dissertation submitted to the Graduate Faculty in Biology in partial
fulfillment of the requirements for the degree of Doctor of Philosophy
The City University of New York.**

1988

II

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ABSTRACT

STUDIES ON THE TRANSFORMATION OF *Escherichia coli* K-12

by

DIONYSIOS LIVERIS

Adviser : Professor Sharon D. Cosloy

The transformation and transfection processes are similarly affected by the same set of conditions in *Escherichia coli*. The chemical oxidation of the outer membrane LPS of recipient cells with periodate does not reduce either transformation or transfection, while the exposure of the recipients to proteolytic enzymes before the induction of competence, greatly inhibits transformation. The inability of vitamin B12 to compete with plasmid transformation suggests that the B12 uptake protein *btu* B is not a DNA uptake "channel". The association of both plasmid and phage DNAs with the polyamines spermidine and putrescine does not enhance either transformation or transfection. Transfecting phage DNAs compete very efficiently with each other and with plasmids for DNA uptake sites available on the recipient cells, while chromosomal DNA competes with transfecting DNA only by means of its mass. Recipient cells recovering from temporary protein synthesis inhibition, caused by either chloramphenicol, or tetracycline, show in the first 30 min of recovery 10-100 fold increases in both transformation and transfection efficiencies. These increases are perhaps the result of the larger number of recipient cells that are able to effectively establish the incoming DNA molecules.

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BACKGROUND

Transformation in bacteria is a gene transfer mechanism involving the incorporation of free extracellular DNA into recipient cells . The transforming "naked" DNA molecules may be produced either by natural cell lysis , or can be chemically extracted and purified from donor cells . When this extracellular DNA is of viral origin , its incorporation into recipient cells results in their transformation-infection or transfection , and the production of new viral progeny . In transfection the intact phage genomes are purified from isolated viral particles . The efficiencies of both transformation and transfection can be measured as the ratio of the number of transformed colonies formed on selective media , or as the number of infective centers (plaques) produced on a "lawn" of phage-sensitive indicator strain , to the total number of viable recipients.

Both transformation and transfection are powerful tools for molecular genetics and genetic engineering . The importance of transformation and transfection arises from the fact that isolated DNA molecules can be manipulated and modified *in vitro* , and then assayed for biological activity by either process . These processes are fundamental in the understanding of the steps involved in genetic recombination , since DNA molecules undergoing recombination can be isolated at any point , characterized and assayed .

Transformation is a complex process which requires the occurrence of a number of cellular events in bacteria . In order to simplify the study of transformation , most studies have assumed that this process is divisible into two distinct phases , DNA uptake and DNA establishment . During uptake , DNA molecules of considerably longer length than the overall length of a cell are able to enter into the cytoplasm of recipient bacteria . In a number of organisms the uptake initially results in the double stranded cleavage of the DNA into smaller fragments . In *E. coli* however , it is believed that this uptake must involve some type of DNA condensing process (Hanahan , 1987) After uptake , the efficient production of transformants requires the cellular establishment of the incoming DNA molecules . The mechanism of DNA establishment in recipient cells is currently unknown .

Transformation has been extensively investigated , but the majority of these studies has been focused on the naturally transformable gram-positive bacteria *Streptococcus pneumoniae*, *Bacillus subtilis*, and the gram-negative *Haemophilus influenzae* (Fox , 1978 ; Low and Porter , 1978 ; Graham and Istock , 1978) . Not all strains of these bacterial species are able to efficiently transform . In *B. subtilis* , the special strains designated "168" or "Marburg" and their derivatives are transformable , while in *H. influenzae* only 4 out of 25 strains designated "Garf", "Parker", "Feld", and "Nine", show efficient transformation (Stuy , 1985) . Similar strain specificity in transformation has been reported in other well studied bacterial genera including *Streptomyces*

(Ledoux et al . , 1976) , *Azotobacter* (Page , 1982) , *Micromonospora* (Tirgari and Moseley , 1980) , *Mycobacterium* (Norgard and Imaeda , 1978) , *Erwinia* (Salmond , 1985) , *Pseudomonas* (Ingraham et al . , 1984) , and *Neisseria* (Juni and Heym , 1980) . In these naturally transformable bacterial strains , transformation is part of their normal life cycle and the genes required for this process are most likely encoded in their chromosomes .

Naturally transformable bacteria have evolved mechanisms for the efficient incorporation of DNA into their genomes . It is believed that the different bacterial genera employ one of two different mechanisms for the uptake of DNA (Smith et al . , 1981) . One mechanism typical of *Streptococcus* and *Bacillus* , and another typical of *Haemophilus* and *Neisseria* . Recent reexamination of the mechanism of transformation of *Haemophilus* and several other bacteria however , suggests that the mechanism of DNA uptake in *Haemophilus* may not be different from that of *Streptococcus* (Stewart and Carlson , 1986) .

The uptake and subsequent establishment of DNA molecules is only possible in physiologically "prepared" cells . This inherited , but transitory ability of naturally transformable cells was coined "competence" by Tomasz (Tomasz , 1969) . The number of competent cells in bacterial cultures depends on the species , the particular bacterial strain and the method of competence induction .

Organisms such as *Escherichia coli* and *Salmonella typhimurium* that

do not possess the special physiological machinery needed for spontaneous transformation , can be made transformable by physicochemical methods (Mandel and Higa , 1970 ; Lederberg and Cohen , 1974) . Transforming DNA can be incorporated into these organisms only after their cell envelopes have been rendered permeable with either some chemical , or enzymatic treatment . There are currently several artificial methods of transformation applicable to both *E. coli* and *S. typhimurium* (Lederberg and Cohen , 1974 ; Davis et al . , 1980 ; Hanahan , 1983 ; Hanahan 1985) . These methods were developed in *E. coli*, because this organism is well known genetically and biochemically , and because these methods are directly applicable in genetic engineering and gene analysis .

Competence development

Bacterial competence is a general term describing the transitory ability of some bacterial cells to interact and irreversibly bind exogenous DNA in order to produce genetically altered recipients . The extent of competence in a particular recipient is measured by the number of transformants , or transfectants that may be generated by either process . Since the above definition of competence also involves the establishment of DNA inside a recipient cell , it is not clear whether competence for transformation with chromosomal DNA refers to the same physiological condition of the recipients as competence for transformation with plasmids , or transfection with phage

DNA . This question arises in view of the fact that the cellular requirements for the establishment of these topologically different DNA molecules inside recipient cells are different . Chromosomal DNA requires genetic recombination into the recipient's genome , while plasmids and phage DNAs require cytoplasmic establishment . The ambiguity in the definition of competence is particularly apparent in the case of transfection , which requires neither host recombination , nor the ability of the host to form colonies . In *E. coli* , experimental evidence suggests that recipient cells which become competent for transformation are also competent for transfection (Berg et al . , 1974 ; Benzinger, 1978) . This has been also confirmed in studies involving transformation and transfection in *B. subtilis* (Riva and Polcinelli , 1968) .

There are several hypotheses about the nature of the competent state in naturally transformable bacteria . These range from the ones that postulate that competence is rather a common condition resulting from the acquisition of a DNA transport system associated with the constitutive proteins of DNA metabolism , to the ones that hypothesize that competence is an unusual event of nucleic acid metabolism requiring the induction of special proteins usually not present in the cell in sufficient quantities (Morrison and Baker , 1979) . The limited experimental evidence currently available on this subject points to the latter as the more likely mechanism of competence (Morrison and Baker , 1979) . Since in both naturally transformable gram-positive and

gram-negative bacteria competence usually develops during the transition from exponential to stationary growth phase , it has been recently speculated that competence in these organisms might have evolved from physiological conditions in which DNA itself might have been sometimes used as the source of nutrients and precursors (Stewart and Carlson , 1986) .

Various members of the genus *Streptococcus* and *Bacillus* become competent by cellular secretion of small , diffusable and species-specific protein competence factors (CFs) (Pakula and Walczak , 1963 ; Tomasz and Mosser , 1966 ; Felkner and Wyss , 1964 ; Streips and Young , 1971) . The mechanisms of action of the various CFs are currently unknown , but noncompetent cells are able to become rapidly competent when exposed to isolated CFs . In *S. pneumoniae* CF is a 5,000 - 10,000 dalton protein , with a hormone-like action (Tomasz and Hotchkiss , 1964 ; Tomasz , 1965 ; Tomasz , 1970 ; Leonard and Cole , 1972 ; Stewart and Carlson , 1986) . This CF induces competence in nontransforming cultures when its effective concentration reaches a critical level . This occurs when the concentration of CF secreting cells is about 10^8 cells/ml (Tomasz and Hotchkiss , 1964) . At this cell density , CF can be isolated from the supernatants of the cultures . The exposure of noncompetent cells to purified CF causes virtually all cells of a *S. pneumoniae* culture to become competent within 10-20 minutes , and to remain in this condition of "temporary differentiation" for an additional 20 minutes , after which competence rapidly declines (Morrison and Baker ,

1979 ; Raina and Ravin , 1981 ; Morrison et al . , 1982) . Competent *S. pneumoniae* show a substantial increase in the length of their cellular chains (Grist and Butler , 1981) . Pulse-labelling studies of competent cells have shown that protein synthesis in these cells is limited to about 11-14 competence-induced proteins (CIPs) ranging in molecular weights from 14,500- 52,000 daltons . Of these CIPs however , only the 19,500 dalton protein seems to be absent from noncompetent cells (Morrison and Baker , 1979 ; Vijayakumar and Morrison , 1986) . The exact function of this major competence-specific protein is currently unknown .

In *E. subtilis* competence appears after a nutritional "shift-down", in which cells are moved from a relatively rich growth medium to a minimal one . This technique brings the cells into a state of "biosynthetic latency", causing the development of competence in about 15% of the viable cells for about 3 hours (Nester , 1964 ; Spencer and Herriott , 1965 ; Venema , 1979 ; Raina and Ravin , 1981) . During this time , several competence-specific or *com* genes are induced and are expressed producing proteins that are apparently required for the binding and uptake of transforming DNA (Dubnau et al . , 1987) . Competent cells of *E. subtilis* are distinguishable from noncompetent ones both physically and physiologically . Based on physical differences , competent cells can be separated from the noncompetent ones by centrifugation on either Renografin , or sucrose gradients (Cahn and Fox , 1968 ; Singh and Pitale , 1967) . The competent cells are less dense , and

contain a single nucleoid , while the larger more dense cells with multiple nucleoids are usually not competent (Dubnau et al . , 1987) . The induction of competence by nutritional "shift-down" treatments of this organism also induce sporulation. Previous studies had suggested that sporulation and competence in *B. subtilis* proceeded independently and in succession (de Lencastre and Piggot , 1979) . However , recent experiments indicate that genetic control of competence is closely linked to the control of sporulation (Dubnau et al . , 1987) . Physiologically competent cells of *B. subtilis* do not replicate , nor synthesize DNA , but they produce and release a positively charged small protein which has properties similar to the CF of *S. pneumoniae* (Akrigg and Ayad , 1970) . Whether this protein has the same function in *B. subtilis* as the CF in *S. pneumoniae* is unclear .

The extent of competence in *B. subtilis* can be regulated by the presence or absence of specific nutritional signals in the culture media (Dubnau et al . , 1987) . While competence cannot be induced in rich media such as LB , certain amino acid mixtures in minimal medium supplemented with glucose are able to either minimize , or maximize the level of cellular competence in *B. subtilis* cultures . The amino acids that interfere with the development of competence in this organism are : alanine , glutamic acid , proline , leucine , and isoleucine , while amino acids that increase its level of competence are : arginine , histidine , threonine , glycine , lysine , and methionine (Erickson , 1970) . The most effective individual amino acid in stimulating competence

in *E. subtilis* is arginine (Lopez et al . , 1975 ; Dr. Rudner personal communication) . This study investigated whether a similar response to the above amino acid mixtures could be elicited in *E. coli* transformation . The basis of this investigation has been the recent suggestion that competence in *E. coli* may have a physiological component (Reusch et al . , 1986) .

The mechanism of induction of competence by CFs are unknown , but when CFs interact with noncompetent cells of *S. pneumoniae* , they seem to bind to specific surface antigens , whose immunological blockage by specific antibodies usually renders the recipient cells noncompetent (Tomasz and Beiser , 1965) . The identities of these cell surface antigens are presently unknown , but the binding of CFs to them , induces several changes in cell physiology and membrane structure . The cell walls of both *S. pneumoniae* and *E. subtilis* become porous due to increased autolytic activity of various amidases associated with the induction of competence in these cells . How the binding of CF , which has no apparent enzymatic activity causes the induction of such autolytic activity is unknown . As a result of the enzymatic erosion of the cell envelope during competence induction , competent cells are osmotically fragile and allow extensive enzymatic leakage of cytoplasmic enzymes into the medium (Young and Spizizen , 1963 ; Ephrussi-Taylor and Freed , 1964 Coleman , 1967) . A similar condition has been observed in *S. sanguis* , where the binding of CF activates an autolytic enzyme whose limited nicking of the peptidoglycan

sacculus at the cellular equator is apparently required for both DNA uptake and cell growth (Tomasz and Horne , 1985) . It is conceivable that external DNA may enter into recipient cells at sites where the cell wall is autolyzed by cellular enzymes in order to allow newly synthesized peptidoglycan to become integrated into the envelope (Smith et al . , 1981) .

Gram-negative bacteria have in addition to the cytoplasmic membrane and murein cell wall , an outer lipopolysaccharide membrane . This outer membrane (OM) is the first selective barrier of these cells to the environment . All substances including DNA , that enter into the cells must traverse this barrier . Despite this additional obstacle , very competent cultures of *H. influenzae* , *H. parainfluenzae* , and *N. gonorrhoeae* may be obtained by nutritional "down shifting" of actively growing cultures (Spencer and Herriott , 1965) . In these bacterial species competence induction is species-specific and may be internally regulated , since no external CFs have yet been isolated .

H. influenzae is able to take up DNA when exposed to various competence inducing factors , such as inosine , lactate and cAMP (Ranhand , 1969 ; Wise et al . , 1973) . Compounds such as valine , periodate , and NAD seem to inhibit the induction of competence in this species (Ranhand and Lichstein , 1966 ; Ranhand and Lichstein , 1969) . The genetically closely related species of *H. parainfluenzae* cannot be made competent by the above agents , but only by allowing the culture to become

saturated by overnight growth (Barany and Kahn , 1985) . In *N. gonorrhoeae* only pilated cell types seem able to become competent (Biswas et al . , 1977) . Competence in both *H. influenzae* and *H. parainfluenzae* is transitory and is kinetically linked to the production of about 10-20 specialized "transformasomes" on their surfaces (Kahn et al . , 1982) . These unique structures are membranous outgrowths specifically involved in both DNA binding and uptake (Kahn et al . , 1982) . The mechanism of this transformasome-mediated DNA uptake is unclear , since binding of DNA to the transformasomes triggers their withdrawal back into the cytoplasm in *H. parainfluenzae* , but not in *H. influenzae* (Barany and Kahn , 1985) . In addition to the production of transformasomes and the synthesis of several inner and outer membrane proteins , physiologically competent cells show a general stimulation of cellular recombination activity (Zoon et al . , 1976) .

Enteric bacteria such as *E. coli* and *S. typhimurium* in which the OM normally shields the cells from the lethal action of bile salts and digestive enzymes secreted in the alimentary canals of animals (Nikaido and Yaara , 1985), can be artificially induced for competence (Mandel and Higa , 1970 ; Lederberg and Cohen , 1974) . The application of various transformation protocols somehow render both the OM and the cytoplasmic membranes of a relatively small group of cells permeable to exogenous DNA . Unlike naturally transformable species , competence in *E. coli* refers to a unique physiological condition that can be repeatedly attained only by the same

group of cells (Sabelnikov et al . , 1984 ; Hanahan , 1987) .

The genetic and biochemical nature of competence in *E. coli* is unknown . However , it has been recently speculated that competence may be the exclusive property of only those cells which are successful in the establishment of incoming DNA molecules after their uptake (Hanahan , 1987) . It is believed that this establishment is limited , because multiple copies of a plasmid do not enhance the cytoplasmic establishment of a single one (Hanahan , 1983) . Transformation studies using labeled DNA have shown that only a very small fraction of the incoming DNA is actually involved in transformation (Weston et al . , 1981 ; Sabelnikov and Domaradsky , 1981) . DNA establishment may be a cell cycle effect caused by the transient synthesis of some unstable proteins as suggested by Hanahan (Hanahan , 1987) . These proteins perhaps are normally involved in the intracellular "management" of DNA , ordering and compacting it during replication . Transforming DNAs may employ these proteins for their successful intracellular establishment (Hanahan , 1987) .

In this study the transient nature of competence in *E. coli* was further investigated by the use of several bacteriostatic antibiotics , which when applied during the induction of competence are able to reversibly prevent protein synthesis . If competence in *E. coli* involves the synthesis of some unstable proteins by a small population of cells , as proposed by Hanahan (Hanahan , 1987) , the antibiotic-mediated inhibition of protein synthesis

during competence induction should reduce the transformability of this organism .

Transformation in *E. coli* is very inefficient . Despite the steady progress that has been made toward improving the *E. coli* transformation system in the past ten years , its efficiency is only a fraction of that of the naturally transformable bacteria . One probable cause of this inefficiency is the limited permeability of transforming DNA into recipient cells (Bergmans et al . , 1981 ; Weston et al . , 1981 ; Sabelnikov et al . , 1984) . Another is the presence of several intracellular degradative nucleases , the most active of which had to be eliminated from recipient *E. coli* strains , before transformation with chromosomal DNA became possible (Cosloy and Oishi , 1973a ; Wackernagel , 1973 ; Oishi and Irbe , 1977) .

Competence inducing protocols of *E. coli* were empirically developed and include , freezing and thawing (Markai et al . , 1964) , protoplast formation by lysozyme-EDTA treatment (Young and Sinsheimer , 1967) , osmotic shock (Taketo and Kuno , 1969a) , growth in hypertonic medium , or in the presence of penicillin (Taketo and Kuno 1969b) , cold CaCl_2 treatment (Mandel and Higa , 1970) , and induction by basic polymers (Benzinger , 1977) . The most common and convenient method of effective induction of competence in this organism is the extensive exposure of cells to relatively high concentrations (50-200 mM) of cold CaCl_2 , followed by a short heat

"shock". The cold- CaCl_2 protocol was initially used to transfect *E. coli* with λ phage DNA (Mandel and Higa, 1970). Subsequently, other investigators have used several improved versions of this basic method to transform and transfect *E. coli* with chromosomal, plasmid, and various phage DNAs with different degrees of success (Cohen et al., 1972; Oishi and Cosloy, 1972; Cosloy and Oishi, 1973; Wackernagel, 1973; Enea et al., 1975; Norgard et al., 1978; Dagert and Ehrlich, 1979; Jones et al., 1981; Bergmans et al., 1981; Hanahan, 1983). From these studies it became apparent that different *E. coli* strains respond to various transformational protocols differently, and to obtain the most efficient transformation with a particular strain, the proper recipient-protocol combinations had to be employed (Hanahan, 1985).

The mechanism of competence induction in *E. coli* is unknown, but the transformation protocols probably generate conditions which promote the association of DNA with the LPS layer of the recipients. The polyanionic DNA molecules are able to electrostatically interact with the polyanionic LPS as Hanahan has speculated, only after Ca^{2+} forms an ionic "shield" around some of the LPS phosphate groups (Hanahan, 1987). This shielding effect by Ca^{2+} is more efficiently obtained at low temperatures which promote relative membrane rigidity (Hanahan, 1987).

Calcium is the most efficient divalent cation in promoting the

transformation and transfection of *E. coli* (Weston et al. , 1981 ; Bukau et al. , 1985 ; Benzinger , 1978) . Although there is no clear answer as to why Ca^{2+} is so effective , the high efficiency of Ca^{2+} for the development of competence in this organism strongly points toward molecular interactions which are well suited for Ca^{2+} . The extent of these interactions probably depend on the physicochemical properties of both Ca^{2+} and the LPS component to which it specifically binds . This point is illustrated by the fact that certain strains of *E. coli* with different mutations in their LPS , are more effectively induced for competence by Mn^{2+} or Ba^{2+} rather than by Ca^{2+} (Taketo , 1975 ; Taketo , 1976) . Calcium ions can be easily distinguished molecularly from other naturally plentiful divalent cations such as Ba^{2+} , Sr^{2+} and Mg^{2+} , none of which is normally effective in the induction of competence in *E. coli* . The characteristics which set Ca^{2+} apart from the other divalent cations are the combination of unique atomic size , charge density distribution , as well as the smallest hydration radius and hydration energy (Cotton and Wilkinson , 1972) . Calcium mediated permeabilization of cellular envelopes to DNA may be a general phenomenon , since it successfully permeabilizes other bacterial species such as *S. aureus* , *H. influenzae* , and *S. typhimurium* (Lindberg et al. , 1972 ; Stuy , 1979 ; Lederberg and Cohen , 1974) . Since *S. aureus* lacks the LPS membrane , it is probable that Ca^{2+} treatment also affects the cytoplasmic membrane .

Under physiological conditions , Ca^{2+} is found almost exclusively in the outer envelope of *E. coli* at around 16 nM /mg (dry wt.) of cell membrane (Letellier and Labedan , 1984) . It is possible that the role of Ca^{2+} is there to "organize" the outer leaflet of the OM by electrostatically binding to the fixed polyanionic LPS molecules (Ferris and Beveridge , 1986) . The resulting reduction in LPS charge repulsions probably induces their proper packing and orientation (Ferris and Beveridge , 1986) . Experimental evidence of this role of Ca^{2+} is provided by X-ray crystallographic studies of the LPS structure and of free lipid A , both of which become highly organized in the presence of Ca^{2+} (Labischinski et al . , 1985 ; Lugtemerg and Van Alphen , 1983) . Cytoplasmic Ca^{++} is usually of very low concentration , because is continuously extruded by active calcium efflux pumps (Silver et al . , 1975 ; Tsujibo and Rosen , 1983) . Radiolabeling studies of *E. coli* and ^{45}Ca have shown that Ca^{2+} accumulated in the cells at 0° C . This Ca^{2+} was released when the cells were heat shocked (Silver et al . , 1975) . The accumulation of Ca^{2+} in chilled cells is probably due to the inactivation of these specific carrier-mediated Ca^{2+} pumps (Rosen and McClees , 1974) .

It is very likely that the competence induction regiment in *E. coli* interferes with the normal cellular modulation of Ca^{2+} , resulting in elevated levels of cytoplasmic Ca^{2+} . The increased concentrations of Ca^{2+} on both sides of the bacterial envelope promote at 0° C the formation of a "gel-like" phase in their lipid components (Overath and Trauble , 1973 ; Verkleij , 1975

Overath et al . , 1975) . This is also the case with synthetic membrane vesicles , which require millimolar quantities of cold CaCl_2 to produce solidifying phase transitions (Papahadjopoulos et al . , 1977) . How the presence of solidified lipids in the cell membranes of recipient cells promotes competence for genetic transformation is a matter of speculation . It has been recently proposed that cold-induced membrane crystallization may allow more efficient electrical shielding of the negative charges on the OM by Ca^{2+} (Hanahan , 1987) . High levels of Ca^{2+} are apparently required to maintain the proper "conditioning" of the OM components for DNA uptake (Sabelnikov and Domaradsky , 1981) . This arises from the observation that rapid washing of *E. coli* cells exposed to DNA with non- Ca^{2+} containing buffers reduces transformation by two orders of magnitude , while similar washing with CaCl_2 has no such affect (Sabelnikov and Domaradsky , 1981) .

There are several other observations which suggest that in addition to better shielding , the lipid solidification of the OM may induce competence by a mechanism that involves some Ca^{2+} -mediated OM damage . In certain strains of *E. coli* , about 30% of cold Ca^{2+} -soaked cells release large amounts of lipopolysaccharides and periplasmic proteins , when heat shocked from 0 ° C to 37 ° C (van Die et al . , 1983) . The mechanism of this damage is unclear , but it seems that the electrochemical shielding of the LPS phosphate groups in association with rapid liquid-gel lipid transitions and

phase separations occurring in the bacterial OM during the heat shock, result in localized delaminations of the OM from its LPS outer leaflet (Overath et al., 1975; Harlos and Eibl, 1980; Shimshick and McConnell, 1973; Cullis and Kruijff, 1979). Such damage to the LPS of *E. coli* may be the reason why the application of the cold CaCl_2 -heat shock protocol usually is lethal to 80%-90% of the recipient cells (Oishi and Cosloy, 1972; Bergmans et al., 1981; Sabelnikov et al., 1984). The damage inflicted in the highly ordered OM by the combination of Ca^{2+} and temperature "shocks" during transformation, reportedly reduce its effectiveness as a permeation barrier against various normally excluded molecules (Sabelnikov and Domaradsky, 1981), and allow the loss of important cellular molecules to the environment (Dagert and Ehrlich, 1979; Nikaido and Nakae, 1979). Also the presence of exogenous DNA seems to be a factor in the reduction of viability of recipient *E. coli* cells, by perhaps partially depolarizing recipient cell surfaces (Sabelnikov et al., 1984). Such reduction in cell viability in the presence of DNA has been also observed in *S. pneumoniae* (Grist and Butler, 1983).

Studies of several "rough" LPS mutants of both *E. coli* and *S. typhimurium*, show no clear correlation between the LPS structure and competence (Taketo, 1977; Taketo, 1978; MacLachlan and Sanderson, 1985). There are no significant differences in transformation efficiencies between wild-type "smooth", and mutant "rough" strains of these organisms with plasmid DNA (Taketo, 1972; Bursztyn et al., 1975; Ledeborg and

Cohen , 1974) . This is perhaps because the particular LPS mutations may not sufficiently affect the conformation of the membrane components involved in DNA uptake (MacLachlan and Sanderson , 1985) . In this report the role of the bacterial OM in competence induction in *E. coli* was further studied . However , instead of employing various LPS mutants , the effects of chemical alteration of the normal LPS in transformation and transfection of *E. coli* was investigated .

The role of Ca^{++} in the induction of competence in *E. coli* is perhaps to induce an extensive and tightly coordinated OM rearrangement . The cooperative rearrangements induced by the interaction of Ca^{2+} with LPS at 0°C could change the conformation of the whole OM , and perhaps expose a specific membrane component or site for DNA binding and uptake (Reusch et al . , 1986) . The specific interactions of OM proteins with LPS seems to be of major importance in the induction of competence of *E. coli* (Weston et al . , 1981) . Fluorescent probe studies have shown that the binding of Ca^{2+} to the LPS and LPS-protein moieties of *E. coli* at low temperatures induces unique conformational changes in the OM that neither Mg^{2+} , Tris , nor EDTA can duplicate in the cold (Sabelnikov and Ilyashenko , 1979) . The identities of OM proteins involved in competence are unknown , although to date two possible candidates have been proposed . These are the *ompA* protein and the *btuB* vitamin B_{12} receptor protein (Taketo , 1977 ; Hanahan ,

1983). In this study the effects of external proteases and vitamin B₁₂ on plasmid transformation was investigated. Trypsin and vitamin B₁₂ were added to competent cells, and their effects on plasmid DNA transformation and phage transfection was determined.

Ca²⁺-induced changes in the OM are apparently required, but are not sufficient to render *E. coli* cells permeable to external DNA (Taketo, 1977; Taketo 1978; Van Alphen et al., 1977). However, these changes allow the efficient uptake of small external proteins into the periplasmic space in about 20% of the cells (Bukau et al., 1985). The uptake of DNA into the cytoplasm of recipient cells requires the rapid temperature transition produced by a heat "shock" step. Under optimal conditions, at best only 0.1%--1% of the cells are able to take up DNA and internalize it in the cytoplasm (Sabelnikov and Domaradsky, 1979; Sabelnikov and Domaradsky, 1981; Weston et al., 1981; Hanahan, 1983).

It has been assumed that competence in organisms like *E. coli*, could be only induced physicochemically. However, recent studies suggest, that competence in this organism, as in naturally transformable bacteria, may have a physiologically regulated component (Reusch and Sadoff, 1983; Reusch et al., 1986). This component appears to be poly- β -hydroxybutyrate (PHB) which is synthesized and is incorporated into the cytoplasmic membranes of recipient cells during competence (Reusch et al., 1982;

Reusch et al . , 1986) . It has been proposed , that this carbon storage compound might facilitate the uptake of DNA through the hydrophobic regions of the cell membrane (Stewart and Carlson , 1986) .

In the last five years , the efficiency of plasmid transformation has been greatly improved over the classic CaCl_2 protocol with of the addition of Rb^+ , or K^+ , dimethyl sulfoxide , dithiothreitol , and hexamine cobalt III-chloride (Hanahan , 1983 ; Hanahan , 1985) to the CaCl_2 solution . The reason for the enhancement of competence of *E. coli* by this more complex protocol is unknown . However , the extreme interdependence of each component in the competence inducing buffer used in this method , suggests that they function in a cooperative manner (Hanahan , 1983) . It is possible that they form a more intricate and stable ionic shield than Ca^{2+} at the hydrophobic-hydrophilic areas of contact formed by the LPS phosphates (Hanahan , 1987) . It is unknown whether this new protocol enhances transformation with all types of DNA , or just plasmids . This question was addressed in this study , where the effect of competence inducing protocols on chromosomal transformation of *E. coli* was investigated .

Several factors are able to influence the efficiency of competence induction in *E. coli* . The method of storage of recipient cells (Hanahan , 1985) , composition of the recipient's growth medium , and the cell densities of the recipient cultures , strongly affect their ability to take up DNA (Saunders

et al . , 1987) . The highest number of competent cells is usually obtained at mid-logarithmic phase , when cell densities are about 10^8 cells /ml (Takeo , 1974 ; Saunders et al . , 1987) . Nutrients that increase cellular growth rates in a particular medium seem to improve transformation (Jones et al . , 1981) . Finally, the pH of the DNA-cell reaction mixture must be within the range of the pH of the culture medium that supports maximal growth which is usually between pH 7.25 and 7.75 (Norgard et al . , 1978) .

Binding and Uptake of DNA

The process of DNA binding and uptake have been investigated in several gram-positive and gram-negative bacteria . When DNA is added to naturally transformable bacteria it rapidly binds to them (Levine and Strauss , 1965 ; Seto and Tomasz , 1974 ; Goodgal and Herriott , 1961) . Competence is not required for such DNA binding , since both competent and noncompetent cells of either gram-positive or gram-negative bacteria , are able to bind DNA to their surfaces (Goodgal , 1982) . This initial nonspecific binding of DNA to bacteria is apparently of ionic nature , and can be easily disrupted by washing the cells , or by DNase digestion (Seto and Tomasz , 1974 ; Dubnau , 1976) . Competent cells are able to convert this "loosely" bound DNA , into irreversibly bound pre-transforming molecules (Smith et al . , 1981) .

There are several cell surface proteins with DNA binding capabilities that have been isolated from both *Streptococcus* and *Bacillus* species (Ceglowski et al . , 1980 ; Smith et al . , 1984) . In *B. subtilis* , a 75 Kdalton dimeric DNA binding factor with endonucleolytic activity has been isolated from the membranes of competent bacilli (Smith et al . , 1984 ; Smith et al . , 1985) . While in *S. pneumoniae* , a single competence-specific protein of 19.5 Kdaltons has been associated with DNA binding in transformation (Morrison and Baker , 1979) . The major membrane-associated endonuclease of *S. pneumoniae* , is apparently not involved in DNA binding , since *end*⁻ mutants which lack the nuclease activity , are still able to bind DNA (Seto and Tomasz , 1974) . In *S. pneumoniae* the number of DNA binding sites is estimated to be about 50 (Fox and Hotchkiss , 1957) . These DNA attachment sites seem to be nonspecific , since heterologous DNAs from different sources are able to bind to them (Ranhand , 1980 ; Tomasz , 1973) .

The DNA molecules which are irreversibly bound to competent cells of *Bacillus* and *S. pneumoniae* are enzymatically fragmented into double-stranded molecules of characteristic size . For *B. subtilis* , these molecules are about 22 Kbs (Dubnau and Cirigliano , 1972) , and for *S. pneumoniae* they are about 8 Kbs (Morrison and Guild , 1973) . Plasmid and phage DNAs are also endonucleolytically processed during entry in the same manner as any other donor DNA is processed in naturally competent cells (Lacks , 1979 ; Contente and Dubnau , 1979) . This results in linearized

plasmids and phage DNAs which do not transform and transfect recipient cells efficiently , because they are unable to replicate and establish themselves in the recipients (Canosi et al . , 1978) . In contrast , the relatively high efficiency of plasmid transformation observed in artificially induced strains of *E. subtilis* suggests that plasmids and phage DNAs may enter into these cells by a different route , which apparently does not involve processing (Smith et al . , 1981) .

The cytoplasmic conformation of donor DNA molecules , in both *E. subtilis* and *S. pneumoniae* is single-stranded (Dubnau and Cirigliano , 1973 ; Plechowska and Fox , 1971) . These single-stranded donor DNA fragments found in the cytosol are apparently selected at random from either strand , and are generated in *S. pneumoniae* by a Ca^{++} -dependent endonuclease (Seto and Tomasz , 1976 ; Morrison and Guild , 1972) , and in *E. subtilis* by a Ca^{2+} - activated endonuclease , and a Mg^{2+} - requiring exonuclease (Scher and Dubnau , 1976 ; Joenje and Venema , 1975) .

In gram-negative bacteria the incoming DNA molecules theoretically have to overcome two naturally imposed obstacles , the outer and inner bacterial membranes . Both of these membranes are chemically and functionally different , and are separated by a more or less continuous compartment termed "periplasmic space". This compartment occupies a substantial volume of the total cellular displacement , and contains in addition to the large number of proteins , the peptidoglycan sacculus in a highly hydrated and

variably cross-linked gelatinous form (Hobot et al . , 1984) . The apparently very small diffusion rates of different macromolecules in the periplasm , further attest to the difficulties that transforming DNA molecules may encounter in penetrating the cellular envelopes of gram-negative bacteria (Brass et al . , 1986) . In *Haemophilus* and *Neisseria* heterologous DNA molecules become bound , but are not taken up into the cytoplasm (Scocca et al . , 1974 ; Dougherty et al . , 1979) . Homospecific double-stranded DNA molecules are taken up very efficiently (Scocca et al . , 1974) , because of the presence of species-specific DNA "uptake sequences" (Danner et al . , 1980 ; Sisco and Smith , 1979) .

In *Haemophilus* , the DNA molecules enter into recipients through competence specific "transformasomes" (Kahn et al . , 1982 ; Kahn et al . , 1983) . DNA molecules properly bound to the transformasomes become physically protected against cellular degradative nucleases , and restriction endonucleases (Kahn et al . , 1983) . The processing of donor DNA from this "protected" state , generates single-stranded DNA molecules (Barany et al . , 1983 ; Pifer , 1986) , which integrate directly into the recipients' genomes during their uptake (Smith and Kahn , 1984 ; Stewart and Carlson , 1986) . The isolation of a large number of plasmids containing deletions following transformation is suggestive that the incoming plasmid DNA in *Haemophilus* is also cleaved by endonucleases during uptake , just like in *E. subtilis* (Albritton et al . , 1981 ; Contente and Dubnau , 1979) , and plasmid

transformants probably arise from the successful repair and recircularization of the plasmid molecules in the cytoplasm . This is further supported by the observation that efficient plasmid and phage transformation and transfection can occur in naturally transformable cells , only when multimeric DNA molecules are used which are capable of recombination to produce unit-length circular plasmids and phages in the host's cytoplasm (Flock , 1978 Stuy , 1980b) .

In the artificially induced transformation system of *E. coli* , the first step in donor DNA transport through the cell envelope , is a non-specific ionic interaction of the DNA molecules with the cell's LPS outer membrane in the presence of excess calcium ions (Deich et al . , 1981) . The initial binding of DNA to recipient cells is followed by a very specific "commitment" step caused by a rapid heat shock treatment which induces a small fraction of the total cell-bound DNA molecules to become resistant to hydrodynamic shear and insensitive to external DNases . This resistance probably arises from the penetration of the DNA into either the cellular periplasm , or the cytosol of the recipients (Weston et al . , 1981) . The populations of cells that take up DNA under these conditions are in some way unique , since they will preferentially take up additional DNA molecules at saturating levels of DNA (Hanahan , 1983) . Under similar conditions of DNA uptake , the OM of *E. coli* also becomes permeable to small proteins (Bukau et al . , 1985) . However , the two processes may have slightly different mechanisms , since protein uptake

into the periplasmic gel , unlike DNA uptake , requires the presence of both calcium and phosphate ions , and can occur without heat shock (Bukau et al . 1985) . The DNA and periplasmic proteins both seem to share some common OM uptake sites , because the uptake of one effectively inhibits the uptake of the other (Bukau et al . , 1985) .

In the *E. coli* transformation system the translocation of DNA through the cell envelope apparently does not require the expenditure of metabolic energy by the recipients (Sabelnikov and Domaradsky , 1981) . The efficiencies of both transformation and transfection are unaffected by the presence of various uncouplers and metabolic inhibitors (Taketo , 1974 ; Sabelnikov and Domaradsky , 1981) . This is in stark contrast to the absolute requirement of cellular energy in the form of either ATP , or a proton motive force for the uptake of DNA into naturally transformable bacteria (Lacks , 1977) . The mechanism that renders donor DNA resistant to external nucleases is poorly understood in *E. coli* transformation , especially in view of the fact that various proteins , including nucleases are apparently able to enter into the periplasmic compartment of cold Ca^{2+} -soaked cells (Brass et al . , 1983) . External DNA molecules become DNase resistant in the CaCl_2 -mediated transformation method , only after a brief heat shock (Bergmans et al . , 1981 ; Weston et al . , 1981) . Similarly , DNAs from phages such as λ and P2 become resistant to external DNases in *E. coli*

spheroplast transfections , only after a heat shock step , suggesting that phage DNA penetrates through the plasma membrane during this step (Benzinger , 1978) . On the other hand , small DNA molecules from phages ϕ x174 and ϕ A transfect competent cells very efficiently in the cold , and any upshift in temperature levels during the cell-DNA interaction , may even reduce the number of transfectants (Taketo , 1974) . How these small phage genomes enter into recipient cells without heat shock is unknown . However , it has been proposed that during the induction of the competence in *E. coli* , some new high-melting (D-) polyhydroxybutyrate PHB domains of the cell membrane , in association with Ca^{2+} may form "pores" that allow the uptake of single-stranded DNA molecules into the cytoplasm (Reusch and Sadoff , 1982) .

Where the donor DNA initially binds on the *E. coli* OM , and how the DNA molecule penetrates both outer and inner cell membranes are currently unknown . An attractive hypothesis put forward (Hanahan , 1983) , proposes that donor DNA molecules may be taken up at the special cellular "zones of adhesion" , previously observed in electromicrographs by Bayer (Bayer , 1968 ; Bayer et al . , 1977) . There are about 400 "zones of adhesion" in the OM of each *E. coli* cell , where the cytoplasmic membrane appears to be fused with the inner phospholipid layer of the outer (LPS) membrane , through "holes" in the continuous peptidoglycan sacculus (Bayer , 1968 ; Muhlradt et al . , 1973) .

LPS-protein components seem to be organized around these "zones" , or

"Bayer's patches", and could serve as controllers for the entrance and exit of various molecules (Osborn and Wu, 1980). The presence of OM proteins at these sites have been confirmed by the observation that several bacteriophages (T1-T7) adsorb exclusively to these areas of the cell envelope (Bayer, 1968). Associated with these sites are also *btuB* proteins normally involved in vitamin B₁₂ transport (Hanahan, 1983). It has been suggested that the vitamin B₁₂ transport apparatus, or perhaps some other cellular uptake system, distorted by the competence inducing conditions, might be responsible for the uptake of DNA into *E. coli* (Hanahan, 1983) (Figure 1). In this study the above proposal was tested by performing competition experiments between B₁₂ uptake and plasmid transformation.

Transformation studies involving known numbers of recipient cells and plasmid DNA molecules, suggest that each *E. coli* cell contains about the same number DNA uptake sites as zones of adhesion. Saturation of these with plasmid DNA does not significantly enhance the chance of a single plasmid in transforming a recipient cell (Hanahan, 1983). Thus plasmid transformation is not a simple probabilistic event, but is a two phase phenomenon initially involving the binding of DNA to both competent and noncompetent recipients, followed by the successful establishment of the plasmid in the competent recipients (Hanahan, 1987).

The mechanism of double-stranded DNA uptake is still unknown in

E. coli , although it appears that during entry , double-stranded fragments of donor DNA are taken up (Yasbin et al. , 1973) . This is supported by the observation that phage DNA isolated shortly after the transfection of *E. coli* with UV irradiated viral DNA , contained thymine dimers on both DNA strands (Strike et al. , 1979) .

Processes following DNA uptake

DNA molecules that are successfully taken up into recipient cells must be stably maintained and become expressed in order to produce transformants . Depending on the nature of donor DNA molecules employed in transformation different intracellular events are required for cells to express their newly "acquired" genotypes . In transfection , after the internalization of phage DNA into the cytoplasm of recipient cells the DNA must replicate and the viral genes must be expressed in order to produce plaques on a phage-sensitive "lawn" of bacteria . Transformation with chromosomal DNA requires recombinational events that replace some of the recipients' genes with homologous ones from a donor . Successful transformation with plasmid needs both the independent cytoplasmic replication of plasmid DNA , and the expression of plasmid encoded genes . It is currently unclear whether the intracellular conditions required for efficient chromosomal transformation are different from those required for plasmid establishment . It has been recently

suggested (Hanahan , 1987) , that the observed differences in chromosomal and plasmid transformational efficiencies in *E. coli* are the result of different DNA organizational requirements that the recipients must fulfill . The more efficient transformation with small plasmids perhaps requires DNA uptake and plasmid replication , while transformation with large plasmids and long fragments of chromosomal DNA may involve "ordering" and compacting of the DNA into chromatinlike structures , a process that perhaps is not very efficient (Hanahan , 1987) .

In *B. subtilis* several recombination-associated genes have been described , some of which are involved in donor-recipient DNA interaction , while others function after the formation of the donor-recipient DNA complex (Dubnau and Cirigliano, 1974 ; Mazza et al . , 1975) . In *S. pneumoniae* the few recombinational mutants documented show reduced levels of transformation caused by deficiencies in a particular ExoV-type DNase (Volvis and Buttin , 1970) . Recombination-associated genes affecting transformation have been also identified in naturally competent gram-negative bacteria. In *H. influenzae* at least two genes are apparently involved in donor-recipient DNA interaction (Caster et al . , 1970 ; LeClerc and Setlow , 1975 ; Taylor and Smith , 1980) . Mutations in either one of these genes drastically reduce the efficiencies of transformation and phage recombination in this organism (Taylor and Smith , 1980 ; Smith et al . , 1981) .

In *E. coli* the efficient recovery of recombinants from chromosomal

transformation , but not plasmid transformation usually requires the *recA* gene product and the deactivation of the *recBCD* enzyme . Mutations in the *recA* gene result in *E. coli* with "recless" phenotypes , in which there is a 10^5 -fold reduction in genetic recombination (Clark , 1973). In recombination the *recA* protein acts as a "synaptase" aligning the donor DNA molecules with the DNA of the recipient's chromosome , while searching for regions of homology between the two DNA molecules (Radding , 1978 ; Radding , 1981) .

In *E. coli* there are three recombinational pathways available for homologous recombination (Clark , 1973) . In wild-type cells the RecBC (*recA*⁺ , *recB*⁺ , *recC*⁺ , *recD*⁺) recombinational pathway predominates and is characterized by the presence of the *recB* , *recC* , and *recD* gene product an ATP-dependent DNase (Exo V) (Dykstra et al . , 1984 ; Basset and Kushner , 1984 ; Biek and Cohen , 1986 ; Amundsen et al . , 1986) . Mutations in either the *recB* , or *recC* locus results in strains that exhibit 1% recombinational activity , and are UV-sensitive . The RecF (*recA*⁺ , *recB*⁻ , *recC*⁻ , *sbcB*⁻) , and RecE (*recA*⁺ , *recB*⁻ , *recC*⁻ , *sbcA*⁻) pathways may become active following the mutational inactivation of the *recBCD* enzyme . The RecE and Rec F genotypes arise from the accumulation of specific suppressor mutations in the RecBC⁻ strains .

The two indirect suppressor mutations of the ExoV⁻ phenotype designated *sbcA* and *sbcB* , neither one of which restores the Exo V activity

are able to restore almost normal recombination to mutants of ExoV (Clark , 1973 ; Barbour et al . , 1970 ; Oishi and Cosloy , 1972 ; Cosloy and Oishi , 1973a ; Cosloy and Oishi , 1973b ; Eisenstark 1977 ; Lloyd and Buckman , 1985) . The *sbcA* locus probably codes for the regulatory protein of Exo VIII (Barbour et al . , 1970) , while mutations in the *sbcB* locus , result in the concomitant abolishment of exonuclease I (Exo I) activity (Kushner et al . , 1971 ; Kushner et al . , 1972) .

In *E. coli* , transformations with chromosomal DNA cannot occur in wild-type cells , but only in recipients possessing the RecF and RecE genotypes , because the Exo V DNase degrades transforming DNA (Oishi and Cosloy , 1972 ; Cosloy and Oishi , 1973a ; Cosloy and Oishi , 1973b ; Sabelnikov et al . , 1975 ; Telander-Muskavitch and Linn , 1981) . In contrast, the ExoV nuclease does not appear to significantly degrade incoming DNA in other genetic transfer systems, such as conjugation and transduction , perhaps because the DNA is either somehow modified , or it enters into recipients via a different route than the transforming DNA . Phage genomes that become sensitive to host nucleases during normal infections , have evolved neutralizing proteins against these enzymes . For instance , phages T4 , T7 , and λ , contain genes coding for Exo V inhibitors (Tanner and Oishi , 1971 ; Unger and Clark , 1972) .

Genetic transformation studies performed by Cosloy and Oishi , on several *E. coli* strains employing the various recombinational pathways have

demonstrated the importance of the intracellular nuclease content of the recipients in chromosomal-mediated transformation of *E. coli* , and have described the conditions that affect this process . The most important of these are the temperature regime of the DNA-cell reaction mixture , and the size of the donor DNA fragments being used . It has been found that the optimal CaCl_2 concentration for competence induction in *E. coli* is 30 mM , while the maximum number of transformants are obtained when the transforming DNA molecules range in molecular weight from 10 to 30×10^6 daltons (Cosloy and Oishi , 1973b) .

Factors influencing the transformation and transfection process .

Polyamines

The presence of a number of polycationic aliphatic polyamines , like spermidine , spermine and putrescine seems to affect transformation of *B. subtilis* (Clark and Leach , 1980) , and transfection of *E. coli* (Benzinger et al . , 1971) . In *B. subtilis* small amounts of polyamines stimulate transformation probably by inducing the synthesis of some competence-specific protein that increases the population of competent recipient cells (Clark and Leach , 1980) . Polycationic compounds also enhance the transfection of a large number of phage DNAs when added to the

cell-DNA reaction mixtures . A 300-10,000 fold increase was observed in transfection of lysozyme-EDTA spheroplasts of *E. coli* with λ DNA in the presence of protamine sulfate (Green et al . , 1966) . There are some indications that protamine sulfate may either facilitate the uptake of DNA or stabilize the spheroplast membranes (Green et al . , 1966) .

An enhancement in the efficiencies of transformation and transfection of *E. coli* spheroplasts was reportedly observed at concentrations of spermidine less than 5×10^{-4} M (Henner et al . , 1973) . However , concentrations of spermidine at 5×10^{-4} M were toxic to the spheroplasts probably because of the oxidation of terminal and /or primary amino groups of cellular proteins into aldehydes (Tabor and Tabor , 1985) .

Polyamines affect many aspects of cellular metabolism , such as protein synthesis , nucleic acid synthesis and conformation (Tabor and Tabor , 1985 ; Gosule and Schellman , 1976) . Polyamines are very effective counter ions of DNA to which they bind very strongly (Kaiser et al . , 1963 ; Tabor and Tabor , 1972) . Polyamine neutralized DNA molecules are apparently protected from shearing forces and heat denaturation (Kaiser et al . , 1963 ; Tabor , 1961) . This stabilizing effect on DNA is probably due to increased tensile strength , or to a more compact tertiary folding of the DNA molecules (Kaiser et al . , 1963) . In this capacity , polyamines seem to be responsible for the folding of viral genomes into viral capsids (Ames and Dubin , 1960) .

Due to the physiological importance of polyamines , and since no studies

have yet been reported on the effects of these compounds on phage transfection and plasmid transformation using intact competent *E. coli* cells instead of spheroplasts, the study of the effects of these compounds on the *E. coli* transformation and transfection system was undertaken.

Oxidizing agent NaIO₄

Although the cell envelope is very important for the development of competence in *E. coli*, its particular component(s) involved in DNA uptake are currently unknown. In this study the effects OM modification by LPS oxidation with NaIO₄, on plasmid transformation and phage transfection in *E. coli* was investigated. Periodate oxidation affects only carbohydrates which contain vicinal alcoholic hydroxyl groups. Both the LPS "O" side chain, and the core polysaccharide of *E. coli* (Figure 10) contain sugars that are labile to periodate oxidation. The periodate treatment of male *E. coli* cells which alters their surfaces causing the inhibition of bacterial conjugation (Sneath and Lederberg, 1961) was the first report of the effect of this reagent on the transfer of genetic information in *E. coli*. In subsequent experiments it was shown that similar periodate treatment also prevents transformation in both competent *B. subtilis* and *H. influenzae* (Polsinelli and Barlati, 1967; Ranhand and Lichstein, 1966). To date, there are no reports on the effects of this reagent on *E. coli* transformation and transfection.

Osmoprotectants.

Non-halophilic bacteria, such as *E. coli* and *S. typhimurium* are able to avoid dehydration and to grow when exposed to media of high osmolarity, by accumulating K^+ and several specific organic solutes in their cytoplasm (Rhoads et al., 1975; Le Rudulier and Bouillard, 1983; Le Rudulier et al., 1984; Gowrishankar, 1985; Landfald and Strom, 1986). These osmoactive molecules allow the stressed cells to increase their internal osmolarities, and to restore their turgor pressures without interfering with other vital cellular functions (Le Rudulier et al., 1984; Barron et al., 1986). The most active of these osmoprotecting compounds are glycine-betaine, and L-proline, which are taken up with increasing efficiency into cells exposed to high osmotic pressure media (Cairney et al., 1985; Perroud and Le Rudulier, 1985; Gawrishankar, 1985).

The osmotic adaptation mechanism in *E. coli* and *S. typhimurium* results in the quantitative alteration of a number of specific OM and periplasmic proteins (Barron et al., 1986). These involve the products of *kds* and *proU* loci (Laimins et al., 1981; Dunlop and Csonka, 1985), as well as the *ompF* and *ompC* porins (Kawaji et al., 1979; Lugtenberg et al., 1976). Similarly the levels of periplasmic alkaline phosphatase (Villarejo et al., 1983), membrane derived oligosaccharides (MDOs) (Kennedy, 1982), and putrescine uptake are also osmoregulated.

In this study , the effects of osmotically induced changes in protein composition of the *E. coli* outer envelope on plasmid DNA uptake were investigated .

Summary of Research Aims.

It is of interest to try to improve the transformation potential of *E. coli* , so the advantages of this experimental tool can be implemented to further understand the mechanisms of DNA uptake and recombination . To this end , several parameters of the *E. coli* transformation and transfection system were investigated in order to characterize them and to enhance their potential . The following is a summary of the studies that were undertaken in the present report :

I . Determination of possible differences between chromosomal and plasmid transformation and phage transfection systems in *E. coli* .

Various transformation and transfection protocols were compared to determine which is the most effective and whether it is equally effective for chromosomal and plasmid transformation , as well as for phage transfection .

The effect of different genetic backgrounds of recipient strains on the efficiencies of transformation and transfection was studied .

II . Investigation of cellular components that may affect *E. coli* plasmid transformation and transfection .

The effect of structural perturbations in normal LPS layers , caused by periodate oxidation , on the transformability and transfectability of several *E. coli* strains was studied . In addition , the effect of proteolytic enzymes on outer membrane proteins of *E. coli* was investigated .

The roles of several compounds with different osmoprotecting activities were assessed for their effects on transformation and transfection . In addition , the cyanocobalamin (B₁₂) transport system was investigated as a possible transport mechanism for transforming DNA .

The effects of different protein synthesis inhibitors on the efficiencies of transformation and transfection in *E. coli* , was measured

III. Investigations on the role of donor DNA in the transformation and transfection processes .

Donor DNA molecules of different size and topology were assessed

The effects of spermidine and putrescine on transformation and transfection were studied .

MATERIALS AND METHODS

Bacterial strains.

All bacterial strains used in this study were derivatives of *Escherichia coli* K12, and are listed in Table 1 . All phage strains and plasmids are listed in Table 2 .

Media .

LB broth contained per liter 10 g tryptone (Difco) 5 g yeast extract (Difco) and 5 g NaCl . SOB broth contained 20 g per liter tryptone , 5 g yeast extract , and 10 mM NaCl , 2.5 mM KCL , 10 mM MgCl₂-10 mM MgSO₄ . TB broth was LB broth , without yeast extract . Penassay broth was prepared medium (Difco-Penassay medium III) in 1 liter of water and was routinely supplemented with 40 µg /ml of thymine .

TB agar and LB agar plates were prepared from corresponding broths with the addition of 1.5 % (w/v) agar (Difco) . LB -ampicillin plates contained 50 µg of ampicillin per ml , while tetracycline plates had 10 µg of tetracycline per ml . Both antibiotics were added as crystals after sterilization of media by autoclaving . All antibiotic containing plates were stored in the dark at 4° C , for no longer than a week . "Soft overlay" agar was made from TB broth with the addition of 0.65% (w/v) agar , supplemented with 10 mM MgSO₄ .

Table 1

Escherichia coli K-12 strains.

<u>Strain</u>	<u>Relevant Genotype</u>	<u>Source or Reference</u>
C600	F ⁻ , <i>thr</i> 1, <i>leu</i> B6, <i>lac</i> Y1, <i>thi</i> 1, <i>ton</i> A21, <i>sup</i> E 44 .	Appleyard , 1954
HfrC6	<i>end</i> A (endonuclease I negative) .	H. Hoffman-Berling
HB101	F ⁻ , <i>rec</i> A13, <i>pro</i> A2, <i>lac</i> Y1, <i>rps</i> L20 (Sm ^r), <i>mtl</i> 1, <i>sup</i> E44 .	Boyer and Dussoix , 1969
AB1157	F ⁻ , <i>arg</i> E3, <i>thr</i> 1, <i>pro</i> A2, <i>lac</i> Y1, <i>his</i> G4, <i>leu</i> B6, <i>thi</i> 1, <i>mtl</i> 1, <i>str</i> 31, <i>tsx</i> 33 .	B. Low
JC7623	F ⁻ , <i>rec</i> B21, <i>rec</i> C22, <i>shc</i> B15, <i>shc</i> C201, <i>arg</i> E3, <i>thi</i> 1, <i>pro</i> A2, <i>lac</i> Y1, <i>his</i> G4, <i>thr</i> 1, <i>leu</i> B6 .	Horii and Clark , 1973
JC5176	F ⁻ , <i>rec</i> B21, <i>rec</i> C22, <i>shc</i> A6 .	Barbour et al . , 1970
W3110	F ⁻ , wild type .	F. Imamoto .
SC401	F ⁻ , <i>rec</i> B21, <i>rec</i> C22, <i>shc</i> A6, <i>trp</i> AB ⁻ , <i>thi</i> -1. (derived from JC5176) .	This Laboratory.
SC403	same as SC401 , but <i>pur</i> F.	This Laboratory .
MO635	<i>rec</i> B ⁺ , <i>rec</i> C ⁺ , <i>shc</i> A ⁺ , <i>thi</i> -1, <i>gal</i> / K2 .	Barbour et al . , 1970

Table 2

Plasmids and phages

<u>Plasmids</u>	<u>Size</u>	<u>Phenotype or Genotype</u>	<u>Source or Reference</u>
pBR322	4.3 Kb	Amp ^r , Tet ^r , colE1 origin.	Bolivar et al . , 1977
pCY35	15.2 Kb	Amp ^r , ~~leu~~ ABCD , colE1 origin .	Squires et al . , 1981

Phages

λd leu	leu AB .
φ80 d trp	Trp E-A .
λ virulent	Non lysogenic in <i>E. coli</i> .
φ80 clear	Non lysogenic in <i>E. coli</i> .
T7	
BF23	Specific for <i>Btu</i> B protein .

Defined media used in this study were M9 and M63 , and Minimal phosphate medium . M9 minimal medium was prepared according to Kellenberger and Sechaud , by adding 7.0 g of Na_2HPO_4 , 3.0 g KH_2PO_4 , 1.0 g NH_4Cl , 0.5 g NaCl and 0.8 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, in 1.0 liter of double distilled water . Following sterilization , the medium was supplemented with 0.2% (w/v) glucose , 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 $\mu\text{g/ml}$ thiamine and 0.2% (w/v) casamino Acids (Difco Laboratories , Detroit Mich .) (Kellenberger and Sechaud , 1957.

M63 media contained 246 mg/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 13.6 g /liter KH_2PO_4 4.0 g/liter $(\text{NH}_4)_2\text{SO}_4$, 0.5 g/liter $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and thiamine (100 $\mu\text{g/liter}$) supplemented with 2g/liter glucose and 0.2% Caseamino Acids (Difco) .

Minimal phosphate medium was made as described by Davis and Mingioli , supplemented with thiamine (1 $\mu\text{g/ml}$) , glucose (0.5%) , and required amino acids (40 $\mu\text{g/ml}$. Minimal agar was made by adding agar at a final concentration of 1.5 % .

Chemicals and Reagents .

All chemicals were obtained from either Sigma Chemical Co. , Fisher or Baker Chemical Co. . Radioactive ^3H thymidine (specific activity 20 Ci/mMol) and ^{14}C leucine (specific activity 54.4 mCi/mMol) were obtained from New England Nuclear .

Buffers and Solutions.

TE buffer contained 1mM $\text{Na}_2\text{-EDTA}$, 10 mM Tris-hydrochloride pH 7.4 . T7 buffer contained 10 mM Tris-hydrochloride pH 7.4 , 1 mM MgCl_2 , and 500 mM NaCl . SSC buffer contained 15 mM NaCl and 1.5 mM $\text{Na}_3\text{-citrate}$. SM phage storage buffer contained 100 mM NaCl , 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50mM Tris-HCl pH 7.5 , 0.01% gelatin . TEB electrophoresis buffer contained 0.089 M Tris (pH 8.0) 0.089 M boric acid , and 0.002 M EDTA (pH 8.0) .

A stock solution of 10mM sodium periodate (Fisher Scientific Co.) was made in double distilled water, adjusted to pH 7.4 with 0.1M NaOH , and filter sterilized using a $0.45\ \mu$ Nylon filter (MSI Co. Honeyoke Falls , N Y) . This solution was stored in the dark at 4°C , for no longer than one week . The activity of the solution was checked before each experiment , with a glucose solution and Schiff reagent . The appearance of purple color at 10^{-4} dilution of the glucose solution was judged satisfactory for the reagent's activity .

Phenol purchased from Baker Chem . Co. was glass distilled under N_2 gas and was stored at -20°C in the dark . When required , a small amount was removed and saturated with 1M Tris-HCl , until its pH was near neutrality . The saturated phenol was stored at -20°C in the dark and 0.1% 8-Hydroxyquinoline was added as an antioxidant .

Pronase stock solutions were made as 10 mg/ml in 0.1XSSC buffer , and

were heated at 70°C for 10 min . These stock solutions were stored at -20°C for one week after which they were discarded .

RNase A stock solutions were made as 2 mg/ml in 0.1 M NaCl-0.01M Na-acetate buffer pH 5.2 . The DNase activity of this enzyme was removed by a 10 min heating at 80°C . The enzyme stock solutions were stored at -20°C for no longer than 2 weeks .

The various polyamine compounds employed in this study were purchased from Sigma . Stock solutions of spermidine , spermine , and putrescine (100 mM) were made in sterile water and stored at -20° C .

Stock solutions of the osmoprotectants proline and glycine-betaine were made in sterile water, as 100 mg/ml and 0.5 mM , respectively . Both were autoclaved and stored at room temperature (22° C) .

Isolation of transforming and transfecting DNAs .

Chromosomal DNA . Chromosomal DNA was obtained from either strains HfrC6 , or W3110 by a modified method of Marmur (Marmur , 1961) . Cells grown to early stationary phase were collected from 1 liter cultures in LB by centrifugation at 8 ,000 rpm for 10 minutes in a prechilled GSA rotor (Sorvall) . The pellets were gently resuspended in 25 ml of cold NaCl (0.1 M)-EDTA (0.1 M) pH 8.5 solution , and the cells were lysed by adding 500

$\mu\text{g/ml}$ pronase for 2 hours at 42°C , followed by the addition of 3 ml of 1 M Tris-HCl, pH 9.0, and 3 ml of 10 % SDS. Crude nucleic acid preparations were obtained by extraction of the viscous cell lysate with 35 ml of Tris-HCl buffered phenol pH 7.0 for 20 minutes. The cell extracts were centrifuged at 22°C , for 10,000 rpm for 10 minutes, and the aqueous layers were collected using large-bore serological pipettes. Nucleic acids were precipitated with 2 volumes of 95% ethanol and were collected by spooling them on glass rods. The collected nucleic acids were redissolved in 20 ml of 1/10 SSC containing 5 mM EDTA. After the DNA was completely dissolved, 1/10 volume of 10X SSC was added to bring the concentration up to 1X SSC, and 25 $\mu\text{g/ml}$ of RNase A (Worthington) was added. The DNA was incubated for 30 minutes at 37°C , and the RNase digestion was terminated by increasing the concentration of the DNA solutions to 0.1 M Tris-HCl. The enzyme was removed by a second phenol treatment. Traces of phenol were removed by 2 or 3 equal volume washings with water-saturated ethyl-ether. Large molecular weight chromosomal DNA from each preparation was precipitated with 2 volumes of 100% ethanol, spooled on glass rods, and was dissolved in an appropriate volume of either SSC or TE buffer. DNA in this form was stored at 4°C with a drop of CHCl_3 for more than a year.

Plasmid DNA. Plasmid pBR322 and pCY35 DNA was isolated by a modified alkaline denaturation method based on the protocol of Birnboim and

Doly (Birnboim and Doly ,1979) . 1 liter LB cultures of strain C600 harboring either plasmid were grown for 4 hours at 37°C, in the presence of 50 µg/ml ampicillin . Plasmid amplification was accomplished by adding 170 µg/ml chloramphenicol and overnight growth at 37°C . The cells were chilled for 10 min on ice and were centrifuged at 8,000 rpm for 10 min at 4°C . The pellets were gently resuspended in 1/25 of the original volume lysozyme solution (25 mM Tris-HCl pH 8.0 , 10 mM EDTA , 2 mg/ml lysozyme , 50 mM glucose) , and were left on ice for 30 min . Crude plasmid DNA was isolated by adding 2 volumes of freshly made alkaline-SDS solution (0.2 M NaOH , 1% SDS) , followed by the addition of 3M Na-acetate pH 4.8 , for a final concentration of 1.5 M Na-acetate . After a 60 min chilling on ice , the denatured chromosomal clots were removed by centrifugation at 8,000 rpm for 20 min in a chilled SS34 rotor (Sorvall) . The supernatants were treated with RNase A (Worthington) at a concentration of 10 µg/ml for 30 min at 37°C , followed by phenol extraction which included , extractions once with one volume of Tris-HCl saturated phenol pH 7.5 , once with one volume phenol : CHCl₃ (1:1) and once with one volume CHCl₃-isoamyl alcohol (24 : 1) . Plasmids were precipitated with 2 volumes of ice cold 100% ethanol . The precipitation was enhanced by chilling the solutions at -20°C for 30 min . The DNA was recovered by a 15 min centrifugation at 12,000 rpm , and was resuspended into a solution of 0.4 M NaCl , 6.5% PEG-6,000 (polyethylene glycol) which

was stored overnight at 4°C . The following day , the DNA-PEG mixture was centrifuged for 10 min at 10,000 rpm . After the supernatant was removed , the DNA was washed with 70% ethanol and vacuum dried . The purified DNA was dissolved in appropriate volumes of TE buffer pH 7.4 and stored at 4°C with a drop of CHCl₃ . The physical structure and purity of these plasmid preparations were routinely checked on 0.8% agarose gels , electrophoresed in a standard "minigel" apparatus with Tris-Borate-EDTA (TBE) buffer at 100 volts for 2 hours . The gels were visualized by staining in 0.5 µg/ml EtBr for 1 hour .

Plasmid Miniprep (Birnboim and Doly ,1979) . Plasmid pBR322 and pCY35 DNA were rapidly isolated from 0.5 ml cultures of C600 grown at 37°C overnight in LB containing 50 µg/ml ampicillin . The cultures were centrifuged at 13,000 rpm for 15 sec in a table top Eppendorf microfuge , and the pellets were resuspended in 100 µl of lysozyme solution (25 mM Tris-HCl pH 8.0 , 10 mM EDTA , 2 mg/ml lysozyme , 50 mM glucose) , and were left on ice for 30 min . Plasmid DNA was obtained by adding 200 µl of freshly made alkaline-SDS solution (0.2 M NaOH , 1% SDS) and incubating on ice for 5 min , followed by the addition of 150 µl of 3 M Na-acetate pH 4.8 for a final concentration of 1.5 M Na-acetate . After 60 min chilling on ice , the denatured chromosomal DNA and cellular proteins were removed by

centrifugation at 13,000 rpm for 5 min . 0.4 ml of the cleared supernatants were transferred into 1.5 ml polypropylene microfuge tubes and 1 ml of ice cold 100% ethanol was added , and chilled for 45 min at -20 °C . The plasmids were precipitated by centrifugation at 13,000 rpm for 2 min in a table top microcentrifuge , and were dissolved in 100 μ l of 0.1 M Na-acetate , 0.05M Tris-HCl pH 8.0 . The DNA was precipitated again with 2 volumes of ice cold 100% ethanol and chilling at -20°C for 30 min . The DNA was recovered by a 5 min centrifugation at 13,000 rpm , and after drying in a vacuum desiccator for 20 min was resuspended into 10 μ l of TE buffer pH 7.4 .

Phage DNA . Phages λ and ϕ 80 were grown on strain C600 in LB overnight . 2 liters of C600 culture in a 4 liter flask was grown to a cell density of 4×10^8 /ml and infected with λ or ϕ 80 at a multiplicity of infection of 0.5 . The infected cultures were incubated at 37° C for 5 hr or until cell lysis was detected. The lysates were treated with few drops of CHCl_3 , and the cell debris were removed by a 10 min centrifugation at 8,000 rpm in a GSA rotor . The cleared lysates were fractionated using 33 % (wt/vol) ammonium-sulfate at 4°C for 2 days , and the precipitated viral particles were collected by centrifugation at 8,000 rpm for 20 min . The phages were further purified by resuspending the phage pellets into SM buffer and isopycnicly banding them on preformed CsCl step gradients in a SW 27 rotor at 22,000 rpm for 2 hours .The step gradient contained CsCl solutions of densities (ρ)

1.15 g/ml , 1.45 g/ml , 1.50 g/ml , and 1.70 g/ml . The recovered bands containing the phages were extensively dialyzed against TE buffer pH 7.4 . DNA from these phages was extracted with Tris-HCl saturated phenol by a 10 min slow rotational extraction , followed by centrifugation at 8,000 rpm for 10 min at 22°C . The top aqueous layers were collected and dialyzed extensively against TE buffer pH7.4 until they had A₂₆₀ nm/A₂₈₀ nm ratios of about 1.8 .

Phage T7 was isolated from lysates of C600 , grown in M9 medium supplemented with requirements and glucose . The cultures were grown to a cell density of 2×10^8 /ml and were infected with T7 at a multiplicity of 5 . The growth was continued at 37° C until lysis was complete (about 3 hrs) . Cell debris was removed by centrifugation at 8,000 rpm for 10 min , and the phages were precipitated and concentrated by two consecutive PEG-NaCl fractionations (9% PEG 6,000 , 0.5 M NaCl) at 4°C for 2 days , according to the method of Yamamoto et al . (Yamamoto et al . , 1970) . The phages were centrifuged for 20 min at 5,500 rpm and were resuspended into T7 buffer , without gelatin . The concentrated phages were banded in CsCl spun at 33,000 rpm for 3 hours in a SW40 rotor at 4°C . The bands containing the phage were collected using an 18-gauge hypodermic needle to puncture the centrifuge tubes . DNA from the phages was purified by the same phenol extraction method used for λ (see above) .

Preparation of Radioactive Plasmid DNA . Radioactively labeled

pBR322 DNA was isolated from strain W 3110 / (pBR322) Thy⁻ , His⁻. 0.2 ml of (methyl- ³H) thymidine (specific activity 20 Ci/mMol) was added to 50 ml of exponentially growing bacteria at 37°C in M9 medium supplemented with thymine (10 µg/ml) and histidine (40 µg / ml) . Growth was continued until the culture reached an optical density of 60 at 590 nm in a Klett-Summerson colorimeter . Selective plasmid amplification was induced by the addition of chloramphenicol (final concentration 50 µg / ml) and further incubation for two and a half hours at 37° C . The radioactive DNA was purified by an exact upscaling of the minipreparation method of Birnboim and Dolly (Birnboim and Dolly , 1979) .

The concentrations of the different DNA preparations isolated in this study were calculated spectrophotometrically in a Zeiss spectrophotometer , using their absorbances at 260 nm . Conversions of absorbance readings into actual DNA concentrations were made by applying the standard conversion factor : A_{260nm} of 1.0 corresponds to 50 µg/ml of double stranded DNA in TE buffer (Maniatis et al . , 1983) . Chromosomal , plasmid and phage , (with exception of T7) DNA yields were estimated to be around 0.5 mg/l of culture , while that of T7 was consistently less in the order of 0.15 mg/l of culture . The DNA stock solutions were stored in their concentrated forms at 4°C . Before each set of experiments , the various DNA stocks were diluted in TE buffer to their proper experimental concentrations which were for chromosomal DNA

about 100 $\mu\text{g/ml}$, for plasmids between 0.5 and 2 $\mu\text{g/ml}$, and for phages about 15-20 mg/ml . Purified chromosomal DNA used in transformations was hydrodynamically sheared by repeatedly forcing it through a sterile 21-gauge hypodermic needle attached to a 1.0 ml syringe . This shearing produced transformationally active DNA molecules of molecular weights of $10\text{-}30 \times 10^6$ daltons (Cosloy and Oishi 1973) .

Transformation and transfection methods.

Three transformation methods were employed in this study . The standard cold CaCl_2 method reported by Davis et al . (Davis et al . , 1980) , which is essentially the same as reported by Mandel and Higa for transfection (Mandel and Higa , 1970) . The high efficiency transformation method of Kushner (Kushner 1978) , modified by Hanahan (Hanahan , 1983) . The transformation method of Reijnders et al . (Reijnders et al . , 1979) , modified by Bergmans et al . (Bergmans et al . 1981) .

Standard Transformation and Transfection Method.

Overnight cultures were diluted 1:20 into fresh LB broth and were grown by gentle agitation in a New Brunswick shaker incubator at 37°C . The cell growth was monitored with a Klett Summerson colorimeter using a red filter

(590 nm) . At early exponential growth phase corresponding to cell densities of 55-60 units the cells were chilled on ice and harvested by centrifugation at 5,000 rpm for 5 min in a cold SS34 rotor . The pellets were resuspended into 1/2 original volume ice cold 50 mM CaCl_2 , and were chilled on ice for 30 min. The cells were pelleted again and were resuspended into 1/20 of the original volume 50 mM CaCl_2 . After an additional 30 min chilling on ice , the cells were considered "competent" for transformation and/or transfection . Each cell-DNA reaction mixture containing 0.10 ml of competent cells and 0.10 ml of DNA-TE buffer mixture was placed on ice for 10 min . The mixtures were heat shocked at 42° C for 90 sec , and were placed for 2 min on ice . In plasmid transformations , 0.8 ml of prewarmed LB broth was added to each reaction mixture , followed by a 30 min phenotypic expression of the antibiotic resistant markers at 37°C , before plating out on selective antibiotic plates . In the case of chromosomal DNA transformations , 0.8 ml of TE buffer was added to the mixtures after the heat shock . Recipients transformed with either chromosomal , or specialized transducing phage DNAs , were plated immediately on selective minimal plates Transfectants were also plated immediately on TB plates , using the liquid overlay method , and C600 as indicator strain . Viable cell counts were determined on either minimal medium supplemented with all requirements , or LB . The transformation and transfection frequencies in all three protocols were expressed as number of

transformant colonies or transfected plaques per viable recipient cells .

High Efficiency Transformation Method .

Overnight cultures were diluted 1:20 into fresh SOB medium and were grown at 37°C with moderate agitation until the cell turbidity of the culture reached 55-60 units at 590 nm on a Klett Summerson colorimeter . The cells were chilled on ice for 10 min , and were pelleted by centrifugation at 5,000 rpm for 10 min . The pellets were resuspended into 1/3 volume of TFB buffer , which contained 10 mM morpholinoethane sulphonic acid (MES) , 45 mM $MnCl_2$, 10 mM $CaCl_2$, 100 mM $RbCl$, and 3 mM hexamine cobalt(III) chloride . The TFB buffer was usually stored at 4° C for no longer than one month . After a 15 min chilling on ice , the cells were further concentrated by a second centrifugation step , and were resuspended into 1/12.5 volume of cold TFB buffer . To each 0.20 ml of this cell solution , a stock solution of DMSO-DTT was added to give a final concentration in DMSO-DTT of 3.5% (v/v) per reaction mixture . Dimethylsulfoxide (DMSO) purchased from Sigma was stored at -20 °C in 1.5 ml polypropylene microfuge tubes under N_2 gas . After each use , the opened tube was discarded . Dithiothreitol (DTT) stock solution was 1.25 M in 0.1 M K-acetate pH 6.0 , sterile filtered on a Millipore filter and stored at -20 °C . Following a 10 min chilling on ice , a second equal volume aliquot of DMSO-DTT stock solution was added and the mixtures

were placed on ice again for 10 min. To 0.20 ml of these competent cells, 10 ml of properly diluted DNA in 0.1 X TE buffer was added, and the reaction mixtures were chilled on ice for 20 min followed by a 90 sec heat shock at 42°C. The reaction mixtures were further cooled by placing them on ice for 2 min after which depending on the nature of the donor DNA, either 0.8 ml of prewarmed LB broth was added, or 0.8 ml of TE buffer was added in the case of chromosomal DNA transformation. Phenotypic expression and plating out procedures were described above.

Transformation Method III.

This transformation protocol was a modified version of the procedure of Bergmans et al (Bergmans et al., 1981). In this method, the recipient cells were not treated with cold CaCl_2 , until they were exposed to donor DNA. Overnight cultures were diluted into fresh LB broth at 1:20 dilution. The cultures were grown at 37°C, with slight agitation until they reached cell densities corresponding to 40 units at 590 nm on the Klett Summerson colorimeter. The cells were chilled on ice for 15 min, and after washing with 1/4 volume saline (10 mM NaCl) were resuspended into 0.05 volume of ice cold 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) buffer pH 6.0. 0.1 ml portions of the cell suspensions were mixed with 0.1 ml of DNA-TE buffer mixtures to which, 0.1 ml of a "Ca-Mg" stock solution (150

mM CaCl_2 -150 mM MgCl_2) were added and chilled on ice for 10 min . The reaction mixtures were heat shocked at 42°C for 90 sec , and were subsequently chilled on ice for 10 min after which 0.7 ml of prewarmed LB broth were added . Phenotypic expression and plating out procedures were performed as above .

Transformation and transfection in the presence of NaIO_4 .

Competent cells induced by the standard CaCl_2 method , were exposed to increasing concentrations of buffered NaIO_4 for 10 min on ice . The interactions were terminated by adding sucrose to a final concentration of 1% . After a 5 min chilling on ice , plasmid or phage DNA was added to these cells and the previously described DNA uptake protocol in standard transformation and transfection method was followed .

Chloramphenicol treatment of *E. coli* cells.

An overnight culture of strain C600 was diluted 1 : 20 ml fresh LB broth , and was grown in a in a reciprocating shaker at 37°C . Growth was monitored in a Klett-Summerson colorimeter with a red filter . When the culture entered early logarithmic phase at a reading of about 40 at 590 nm on the colorimeter, one third of the culture was removed into another flask and was further grown under the same conditions until the late logarithmic phase of 60

units on the colorimeter at 590 nm . This culture designated as "control" , was then chilled on ice .

The remaining culture , was treated with a methanolic solution of chloramphenicol (final concentration of 100 µg/ml in 0.2% methanol) . This concentration of methanol had no effect on the growth or on the induction of competence in the cells. The antibiotic-treated cells were reincubated in the shaker for an additional 30 minutes at 37°C , and were harvested by centrifugation (5,000 Xg for 10 min in a Sorvall SS34 rotor) at 22°C . The antibiotic was removed by two washes of equal volume of LB broth , and the cells were resuspended into fresh pre-warmed LB broth . A portion of this culture designated 'before' recovery from the action of the antibiotic , was immediately removed and chilled on ice . The remainder of the culture was further incubated at 37°C on the shaker platform to recover from the bacteriostatic effects of chloramphenicol . This recovery was monitored by the resumption of growth . When the density of the cultures reached a reading of 60 units at 590 nm on the colorimeter, further recovery was stopped by chilling on ice . These cultures were designated as "after" recovery .

All these samples were assessed for plasmid DNA transformation and bacteriophage DNA transfection , as well as for ³H-DNA binding after being made competent by the standard CaCl₂ method of Davis et al . , 1980) . Frequencies of transformation and transfection were expressed as antibiotic

resistant colonies or plaques per viable cell at the end of the expression period .

Binding of ^3H -plasmid DNA to *E. coli* cells.

To measure the amount of ^3H -pBR322 DNA that binds to competent C600 cells , 10 μl of radioactive plasmid DNA were added to competent cells . These mixtures were chilled on ice for 10 minutes , followed by a 90 sec heat shock at 42°C . For the measurement of total ^3H -DNA bound to whole cells , the cells were filtered on a membrane filter (0.45 mm ; Millipore Corp.) , washed with 3 X with 5ml of SN buffer (20% sucrose - 0.15M NaCl) at 22°C . The washed filters were removed from the filtration apparatus and allowed to air dry overnight . The radioactivity retained on the filters , was measured in a LKB tabletop scintillation counter with a scintanlized toluene cocktail (P.P.O /P.O.P.O.P : 12 g / 0.3 g / 3 lit. toluene Fischer Corp.) . The values for total labeled DNA bound presented here , are the average of two independent assays , each read twice for one minute intervals using a single ^3H label window . The amount of ^3H DNA that was bound to cells in a DNase-resistant form was determined by first centrifuging the DNA treated cells in a Beckman microfuge (13,500 X g for 5 min) and washing the pellets with 4 ml of SN buffer. After recentrifugation , the pellets were resuspended into 200 μl of SN

buffer , containing 50 μ g of bovine pancreatic DNase (Sigma Chemical Corp.) in 10 mM MgCl-0.2M Na-acetate pH 5.0) , and chilled on ice for 15 min . After the chilling , the cells were transferred to Millipore filters , washed twice with 4.0 ml of SN buffer and were dried and counted as above .

The amount of labeled DNA incorporated into the cells in a trichloroacetic acid (TCA) precipitable form was determined by the following method . Transformed cells were washed with three 4 ml washes of SN buffer . The washed cell pellets were resuspended into 1 ml of double-distilled water containing 50 mM Tris-HCl (pH 8.0) to which 100 μ g of lysozyme (Sigma Chemical Co.) per ml was added . The reaction mixtures were put on ice for 10 min after which 0.25 ml of ice cold 50% trichloroacetic acid solution was added . The cells were rapidly shaken for 60 sec and further chilled on ice for 10 min . The amount of radioactivity was measured by transferring the contents of each mixture to a standard-grade GFA absorbent filter pad , followed by extensive washing (15ml) of each pad with ice cold 5% TCA solution , and 10 ml of 95% ethanol . The pads were removed , allowed to air dry , and were counted in the scintillation counter .

¹⁴C Incorporation assays.

Levels of protein synthesis in cells transformed with plasmid pBR322 were determined from ¹⁴C labeled leucine incorporation into TCA precipitable

proteins during phenotypic expression after transformation . At the beginning of phenotypic expression , 10 μ l of ^{14}C -leucine (specific activity 54.4 mCi / miviol) were added to 1 ml of LB broth diluted transformation reaction mixtures . After 30 min of incorporation , the cells were harvested by centrifugation at 5,000 rpm for 5 min at 4°C . The pellets were washed three times with SN buffer , and were suspended into 1 ml of cold water containing 100 μ g /ml lysozyme . Following a 10 min chilling on ice , 0.25 ml of ice cold 50% TCA solution was added and thoroughly mixed , the reaction tubes were further chilled on ice for 25 min . The precipitates were collected on standard GFA filters by vacuum suction , were washed with 15 ml of cold 5% TCA , and 10 ml 95 % ethanol . The amount of label retained on each filter , was measured in an LKB liquid scintillation counter .

RESULTS

Comparison of strains and transformation protocols .

The efficiency of plasmid transformation in *E. coli* is dependent on the particular strain and transformation protocol being employed (Hanahan , 1983) . In order to find the optimal recipient-transformation method combinations , several different *E. coli* strains were examined for their abilities to transform with plasmids , using three different transformation protocols . The choice of the particular *E. coli* strains was on the basis of their genetic heterogeneity and transformation proficiency . Plasmid pBR322 and its larger derivative pCY35 were used for all transformation assays . Both of these plasmids are multicopy cloning vectors derived from plasmid ColE1 , and confer ampicillin resistance to their hosts .

The three transformation protocols used in this study included the "standard" $\text{CaCl}_2/0^\circ\text{C}$ method (Davis et al . , 1980) , the "complex" RbCl -organic solvent method (Hanahan , 1983) , and the "rapid" Ca^{2+} - Mg^{2+} -shock method reported by Bergmans et al (Bergmans et al . , 1981) . This last protocol was included in this study because it does not involve protracted treatment of the recipients with $\text{CaCl}_2/0^\circ\text{C}$, which could result in extensive loss of cell viability . Table 3 contains a summary of conditions and factors of the above three protocols .

The efficiencies of transformation obtained with the three protocols were calculated as the ratio of the number transformants obtained per ml per total number of viable cells . Since nonsaturating plasmid DNA levels were used throughout these experiments , the above transformation efficiency values give an estimation of how well individual competent cells take up and establish plasmid DNAs . Table 4 tabulates the transformation efficiencies of five different recipient strains . Among the three transformation methods , the "complex" RbCl-organic solvent protocol was consistently the most effective for all strains tested with both plasmids . The "standard" cold CaCl_2 protocol was less effective in the production of plasmid DNA transformants , while the method of Bergmans et al . was the least effective in this regard . It is unknown whether the three transformation protocols affect the cytoplasmic establishment or some cell surface component during competence induction . That they may affect cell surface components differently is evident from the fact that even though both the "high efficiency" and the "classical" protocols were applied to more or less the same number of cells , the former consistently yielded slightly less viable cells . The substantially lower transformation efficiencies obtained by the "rapid" Ca^{2+} - Mg^{2+} shock method was perhaps because the short pulsing of the recipient cells with the Ca^{2+} - Mg^{2+} mixture before the heat shock induces lower numbers of competent cells .

Comparison of bacterial strains.

Although there are clear differences in transformation efficiencies between the various strains of *E. coli*, some appear not to correlate with any particular locus, or genotype. The highest plasmid transformation frequencies were found with strains SC401 (RecE) and C600. Strains SC401 and MO635 are of the same lineage, but SC401 utilizes the RecE pathway and lacks ExoV, where as MO635 utilizes the RecBC pathway and produces ExoV. Comparison of SC401 to MO635 show that there is about a 100-fold difference in plasmid transformation between these strains, and clearly indicates that ExoV may inhibit plasmid transformation as well as chromosomal transformation. However, strain JC7623, which lacks ExoV, has considerably lower transformational efficiencies than SC401 and C600 which produces ExoV. All these strains are of different lineage, and the results suggest that there are other unknown genetic factors that affect the efficiencies of transformation in *E. coli*.

Table 3

Comparison of transformation protocols in *E. coli*.

Standard Method CaCl ₂ / 0°C	Complex Method RbCl - Org Solvent .	Rapid Method (III) Ca ⁺² - Mg ⁺² Shock
1. CaCl ₂	RbCl, CaCl ₂ , MnCl ₂ , HACo(III)Cl ₃	HEPES (pH 6.4)
2. 0°C	0°C	0°C
3. --	DMSO, DTT	CaCl ₂ , MgCl ₂
4. + DNA	+ DNA	+ DNA
5. 0°C-42°C-0°C	0°C-42°C-0°C	0°C-42°C-0°C

1. Initial solution used to suspend the recipient cells in the competence induction protocols .

2. Incubation temperature of recipient cells ranging from 30-60 min .

3. Factors added during incubation (complex method) or after incubation (Rapid method III) .

4. + DNA designates the addition of DNA to the competent cells .

5. Heat shock methodology .

For details see Materials and Methods .

Table 4

PLASMID TRANSFORMATION FREQUENCIES OF *E. coli*

Strain	Protocol	Plasmid	Ave. Transformation.	Viable Count. X10 ⁸ /ml
	Employed	DNA	Frequency	
C600	I •	pBR 322	3.00 X 10 ⁻⁵	6.22
		pCY 35	9.00 X 10 ⁻⁵	
	II **	pBR 322	7.10 X 10 ⁻⁵	2.70
		pCY 35	1.80 X 10 ⁻⁴	
	III ***	pBR 322	1.60 X 10 ⁻⁷	1.42
		pCY 35	1.15 X 10 ⁻⁶	
	I	pBR 322	7.20 X 10 ⁻⁶	1.85
		pCY 35	1.13 X 10 ⁻⁵	
	II	pBR 322	1.10 X 10 ⁻⁵	1.70
		pCY 35	4.20 X 10 ⁻⁵	
Hb101	I	pBR 322	1.30 X 10 ⁻⁷	1.90
		pCY 35	2.51 X 10 ⁻⁶	
	II	pBR 322	1.82 X 10 ⁻⁶	4.35
		pCY 35	1.30 X 10 ⁻⁶	
	III	pBR 322	3.40 X 10 ⁻⁵	2.30
		pCY 35	4.51 X 10 ⁻⁵	
	I	pBR 322	1.72 X 10 ⁻⁶	7.55
		pCY 35	1.72 X 10 ⁻⁶	
	II	pBR 322	9.20 X 10 ⁻⁵	1.45
		pCY 35	2.14 X 10 ⁻⁴	
JC7623	I	pBR 322	2.70 X 10 ⁻⁴	1.05
		pCY 35	5.33 X 10 ⁻⁴	
	II	pBR 322	4.60 X 10 ⁻⁵	1.05
		pCY 35	4.50 X 10 ⁻⁵	
	III	pBR 322	9.20 X 10 ⁻⁵	1.45
		pCY 35	2.14 X 10 ⁻⁴	
	I	pBR 322	2.70 X 10 ⁻⁴	1.05
		pCY 35	5.33 X 10 ⁻⁴	
	II	pBR 322	4.60 X 10 ⁻⁵	1.05
		pCY 35	4.50 X 10 ⁻⁵	
SC401	I	pBR 322	9.20 X 10 ⁻⁵	1.45
		pCY 35	2.14 X 10 ⁻⁴	
	II	pBR 322	2.70 X 10 ⁻⁴	1.05
		pCY 35	5.33 X 10 ⁻⁴	
	III	pBR 322	4.60 X 10 ⁻⁵	1.05
		pCY 35	4.50 X 10 ⁻⁵	
	I	pBR 322	9.20 X 10 ⁻⁵	1.45
		pCY 35	2.14 X 10 ⁻⁴	
	II	pBR 322	2.70 X 10 ⁻⁴	1.05
		pCY 35	5.33 X 10 ⁻⁴	

Table 4 (continued)

PLASMID TRANSFORMATION FREQUENCIES OF *E. coli*

Strain	Protocol Employed	Plasmid DNA	Ave. Transformation Frequency	Viable Count. $\times 10^8/\text{ml}$
MO635	I	pBR 322	8.32×10^{-7}	2.80
		pCV 35	1.15×10^{-6}	
	II	pBR 322	1.45×10^{-5}	1.02
		pCV 35	1.32×10^{-5}	
	III	pBR 322	7.01×10^{-6}	1.25
		pCV 35	2.00×10^{-6}	

I * Standard transformation protocol refers to that of Davis et al. (Davis et al., 1980).

II ** High efficiency transformation protocol refers to that of Hanahan (Hanahan, 1983)

III *** Bergmans transformation protocol is that of Bergmans et al. (Bergmans et al., 1981).

Transformation frequencies are expressed as ampicillin-resistant colonies formed per total number of viable cells for 10 ng of pBR322 or 30 ng of pCV35 DNA. The average values in Table 4 are derived from at least 3 independent transformation assays with each protocol.

To establish whether the high efficiency RbCl-organic solvent transformation protocol developed for plasmid DNA transformation was able to also enhance transformation with chromosomal DNA, strain SC401(Rec E) was transformed with sheared chromosomal DNA isolated from the prototrophic strain HfrC6, using both the cold CaCl_2 and the RbCl-organic solvent transformation protocols. Strain SC401 was used because it lacks ExoV and is still recombination proficient. The results on Table 5 show that the "classical" CaCl_2 method is better than the RbCl-organic solvent method for chromosomal transformation. The results of these experiments point to the fact that efficient plasmid DNA transformation in *E. coli* does not guarantee efficient transformation with chromosomal DNA.

Although the "complex" RbCl - organic solvent method is clearly the most efficient protocol for plasmid DNA mediated transformation in all strains of *E. coli* tested in this study, its complexity and extreme dependence on reagent purity and condition ruled against its use in the majority of experiments in this study. Instead, the modified CaCl_2 protocol of Davis et al. (Davis et al., 1980) was employed as the standard method of transformation and transfection in most of the experiments reported here.

Table 5

**Effects of Competence Induction Protocols on Chromosomal DNA
Transformation.**

Protocol	Transform. Efficiency (Trp⁺)	Viable Count X10⁸/ ml
Cold - CaCl₂	5.32×10^{-7}	8.40
RbCl - Org. solvent	1.53×10^{-7}	8.15

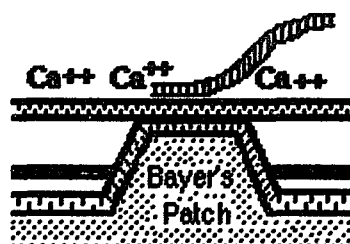
Strain SC401(RecE) was grown in SSA medium with its requirements and was subsequently transformed with the protocols of Davis et al., 1980 and Hanahan, 1983. Transformation was performed with 10 µg of Hfr6 DNA per tube. Transformation frequencies were calculated as the ratio of the number of Trp⁺ colonies obtained per ml of reaction mixture to the total number of viable recipient cells per ml. Trp⁺ recombinants were scored on minimal phosphate plates after 48 hr of incubation at 37° C. The values are the average of two independent trials.

Figure 1

Hypothetical mechanism of transformation in *E. coli*

- I. Attachment of DNA to the surface of competent cells in the presence of Ca^{++} at 0°C .

Chromosomal DNA

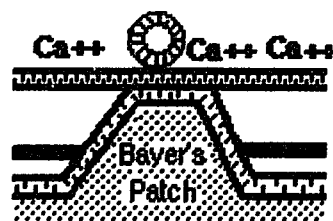


Outer Membrane

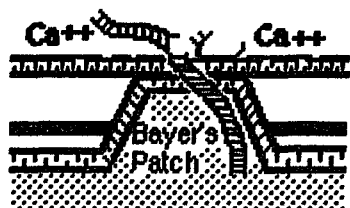
Cell Wall

Cell Membrane

Plasmid DNA



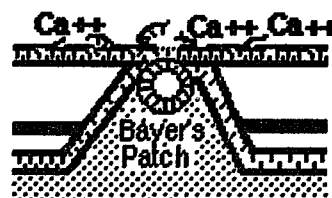
- II. Uptake of DNA during heat shock at 42°C .



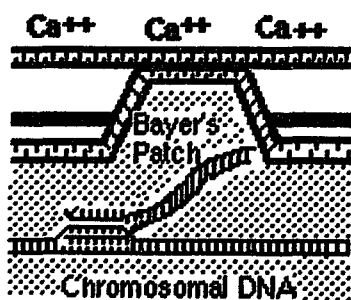
Outer Membrane

Cell Wall

Cell Membrane



- III. Establishment of DNA in the recipient cell.

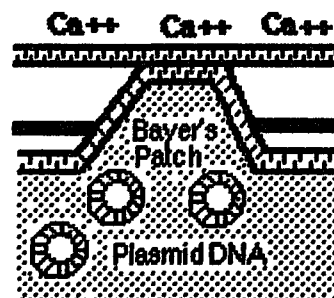


Outer Membrane

Cell Wall

Cell Membrane

Chromosomal DNA



Plasmid DNA

Transformation and transfection kinetics.

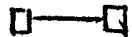
Two previously unaddressed aspects of the kinetics of DNA uptake by competent *E. coli* were analyzed, how rapidly transforming plasmid DNA associates with recipient cells before the heat shock, and what is the state of the DNA in the intercellular environment. Competent cells of strain C600 were mixed with plasmid pBR322 DNA on ice, and at various times during the period of cell-DNA interaction, a sample was removed and heat shocked for 90 sec at 42°C. These samples were subsequently assayed for plasmid transformation as described in Methods. The results in Figure 2 show that the number of transformants increased rapidly with time of exposure to DNA in the cold, suggesting that plasmid DNA is able to effectively associate with competent cells rapidly. Within the first 1 min of DNA-cell interaction on ice, close to 50% of the total number of transformants were obtained when heat pulsed. Maximal numbers of transformants however, were scored only after 10 min of DNA-cell interaction on ice before the heat shock. The exposure of competent cells to plasmid DNA on ice for time periods longer than 10 min did not increase significantly their transformation efficiencies (data not shown). This is in agreement with the suggestion that the transforming DNA rapidly associates with the cellular OM electrostatically (Hanahan, 1987).

Figure 2. Strain C600 was induced for competence by the method of Davis et al. (Davis et al , 1980) At various times indicated during the 10 min chilling on ice with plasmid pBR322 DNA 0.10 ml samples were removed and exposed to a 90 sec heat shock at 42 °C. Transformation frequencies were computed as the number of Amp^r colonies per total number of viable cells The results in Figure 2 are the average of three independent trials .

Legend :

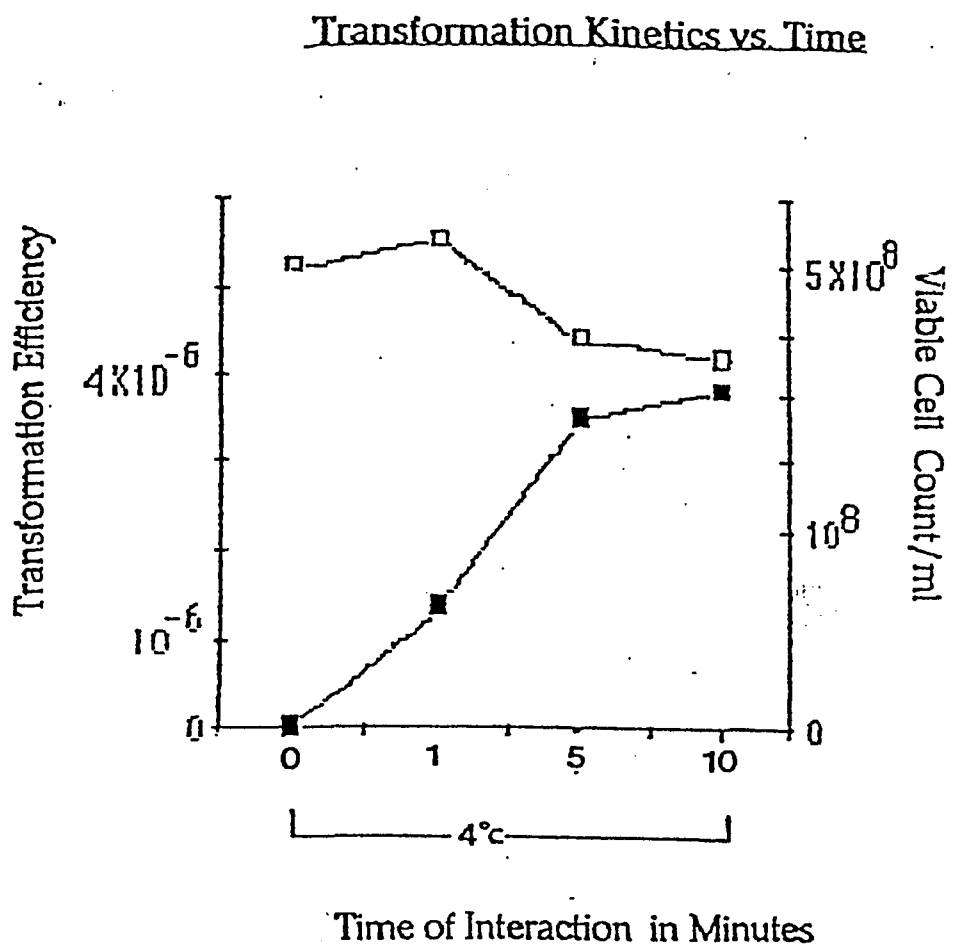


Plasmid pBR322 Transformation Efficiency .



Viable Counts .

Figure 2.



In a related experiment the kinetics of plasmid and phage DNA uptake into intact cells during the standard 90 sec of heat shock was examined . The results of this experiment is presented in Table 6 , which shows that both plasmid transformation and phage DNA transfection follow similar kinetics of DNA uptake during the length of the heat pulse at 42°C . In the first 25 sec , about 25% of the total transformants and more than 50% of the total number of transfectants were obtained . After the 90 sec heat pulse , the presence of DNase did not result in reduced efficiencies of transformation or transfection relative to those of the untreated controls . Similar results have been also seen in *E. coli* spheroplast transfection (Benzinger , 1978) .

Effect of non-transfecting DNA on transfection in *E. coli*.

The process of DNA uptake into competent recipients was further investigated by studying the effect of the presence of increasing amounts of non-transfecting Hfr-C6 DNA on transfection . Previous reports have shown , that non-transforming DNA competed with plasmid DNA transformation , as a function of its mass (Humphreys et al . , 1979 ; Hanahan , 1983) . The experiments reported here show a similar response of transfecting DNA to increasing concentrations of Hfr-C6 DNA . Table 7 shows that transfection efficiencies declined as a function of increasing Hfr-C6 DNA mass .

Table 6**Kinetics of Transformation and Transfection in *E. coli*.**

Length of Heat Shock in sec	Transformation Efficiency X 10⁻⁶	Transfection Efficiency X 10⁻⁶	Viable Count X 10⁸/ml
0	<0.01	<0.01	7.85
25	2.30	2.50	8.00
50	3.40	3.40	8.42
75	4.70	4.80	7.85
90	8.50	4.00	6.65
<u>90 (-DNase)</u>	<u>7.72</u>	<u>4.25</u>	<u>8.05</u>

Strain C600 was grown in LB and was induced for transformation by the protocol of Davis et al. 15 ng of pBR322 and 600 ng of λ vir DNA/0.2 ml reaction mixture was added to 0.1 ml of concentrated cells and the mixtures were chilled on ice for 10 min before the 90 sec heat shock at 42°C. At the various times indicated, samples were taken and 50 μ g of pancreatic DNase was added and incubated on ice for 15 min. Transformation and transfection efficiencies were calculated as previously described. The results are the average of two trials.

Transfection frequencies were not significantly affected over a range of phage DNA/Hfr-C6 DNA ratios of 1:1 to 1:10, but they rapidly declined at

ratios of 1 : 20 to 1: 1000 . These results support the suggestion that transformation and transfection in *E. coli* is independent of DNA uptake , but require successful intracellular DNA establishment (Hanahan , 1983) . The ability of Hfr-C6 DNA to prevent transfection only at relatively high concentrations is perhaps because transfection of a recipient requires the binding and uptake of a single phage DNA molecule , while the inhibition of transfection by nontransfecting DNA requires the efficient blocking by this DNA of all the estimated 200-300 independent DNA uptake sites on a cell (Hanahan , 1983) . The above results also suggest that the DNA uptake sites on the surface of recipients are nonspecific with respect to the type of DNA being bound to them . Whether they are plasmids , or chromosomal DNA their binding to these sites is efficient and rather stable , since once a particular DNA molecule is bound , it cannot be easily outcompeted by other DNA molecules . This point was further investigated by performing competition experiments with two different DNAs . In these experiments , the results of which are shown on Table 8 , transducing phage DNA served as donor DNA for chromosomal transformation . The simultaneous presence of both DNAs in equal concentrations , resulted in the reduction of transformational frequency of either one by about 50% . The frequency of double transformants (Leu^+ , Trp^+) which requires the establishment of both DNAs , was 10-fold less than either DNA selected alone in the presence of its competitor .

Table 7**Competition of Hfr-C6 DNA with ϕ 80 DNA in Transfection**

ϕ 80DNA	Hfr-C6 DNA	ϕ 80/Hfr DNA Ratio	Transfection Effic. $\times 10^{-5}$
100ng	0	---	3.00
100ng	100ng	1:1	4.80
100ng	200ng	1:2	3.50
100ng	500ng	1:5	4.43
100ng	1,000ng	1:10	3.02
100ng	2,000 ng	1:20	1.90
100ng	5,000ng	1:50	1.25
100ng	10,000ng	1:100	0.81
100ng	20,000ng	1:200	0.60
100ng	50,000ng	1:500	0.03
100ng	100,000ng	1:1000	0.01

E. coli SC401 was grown in LB and was transfected with the protocol of Davis et al., 1980

Transfection was done using 100ng of ϕ 80 clear premixed with increasing amounts of Hfr-C DNA. Transfection frequencies were calculated as the ratio of plaques per ml to the total number of viable recipient cells per ml. Strain C600 was used in all platings as indicator strain. Each experiment was repeated three times.

These results support further the suggestion that DNA establishment is the rate determining process in *E. coli* transformation (Hanahan, 1983). In the presence of equal number of two competing DNA molecules, half of the available DNA uptake sites on the recipients are occupied by DNA molecules, the uptake of which produces transformants that are not selected for, and thus are undetected. Since the number of competent cells in a culture is limited, the presence of these non selected transformants would reduce the number of transformants that are selected for by about 50 %. The observed very low efficiency of double transformants is suggestive of the limiting amounts of intracellular factors available for DNA establishment (Hanahan, 1987). The establishment of a single DNA almost excludes the establishment of a second DNA molecule in a recipient.

Cellular Components Affecting Transformation and Transfection.

Effects of NaIO₄ on transformation and transfection of *E. coli*.

The effect of chemical damage of the LPS on transformation and transfection was investigated to determine whether the LPS plays a role in DNA uptake. Several strains of *E. coli* were treated with the oxidizing agent periodic acid and were assayed for plasmid transformation and phage transfection.

Table 8

Effect of Competing DNA on Transformation in *E. coli*

DNA	Average Transformation Efficiency X10 ⁻⁶		
	<u>Leu</u> ⁺	<u>Trp</u> ⁺	<u>Leu</u> ⁺ <u>Trp</u> ⁺
λ leu	8.10	----	----
ϕ 80bp	----	2.56	----
λ leu + ϕ 80bp (1:1)	4.56	1.50	0.10

E. coli SC641 was grown in LB and was transformed with the protocol of Davis et al., 1980

The transformations were using either 100ng of λ leu DNA alone, or premixed with 100 ng of ϕ 80 trp DNA. Transformation frequencies were calculated as the ratio of Leu+ on Trp+ colonies per ml found on tryptophan containing minimal, or leucine containing minimal plates to the total number of viable recipient cells grown on minimal plates containing both amino acids. Each experiment was repeated five times.

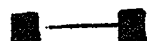
The optimal length of time for periodate treatment of competent cells was determined from a time-course experiment using 10⁻³ M sodium-periodate.

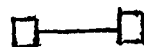
Figure 3 establishes that periodate treatment affected the cells most dramatically in the first ten minutes of exposure . Several strains of *E. coli* were tested for transformation and transfection following exposure to increasing concentrations of periodate . The results in Tables 9 and 10 show that both recipient cell viability , and the ability to transform and transfect are severely reduced in all *E. coli* strains at concentrations above 10^{-3} M of NaIO_4 but below this concentration of periodate , there was only a small reduction of transformation and transfection . *E. coli* strains did not show a reduction of transformability and transfectability at periodate concentrations between 10^{-4} M to 5×10^{-4} M . This is in contrast with the previously observed severe reduction of both of these processes in *B. subtilis* and *H. influenzae* (Polsinelli and Barlati , 1967 ; Ranhand and Lichstein , 1966) in this concentration range of periodate .

The apparent resistance of *E. coli* transformation and transfection to periodate oxidation , provides further evidence that this organism employs a transformation mechanism that is different from that of naturally transformable species which contain a periodate-sensitive stage . These results do not support the speculation that LPS might sterically prevent the uptake of external DNA , since its apparent removal does not enhance transfection or transformation in *E. coli* .

Figure 3. C600 was induced for competence by the cold CaCl_2 method of Davis et al , (Davis et al ., 1960) , and was exposed to 10^{-3} M of buffered Na periodate at 4°C in the dark . At various times indicated , the periodate was neutralized with glucose to a final concentration of 1% , and the cells were removed by centrifugation . The periodate-treated cells were resuspended into 50 mM CaCl_2 , and were transformed with 10 ng of pBR322 and 200 ng λ vir DNA . Amp^r colonies were selected on LB plates supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin , while transfectants were selected on TB plates using TB overlay supplemented with 10 mg/ml MgSO_4 and C600 as indicator strain . Transformation and transfection frequencies were calculated as before . The results in Figure 3 are the average of three independent trials .

Legend :

 Plasmid pBR322 Transformation Efficiency .

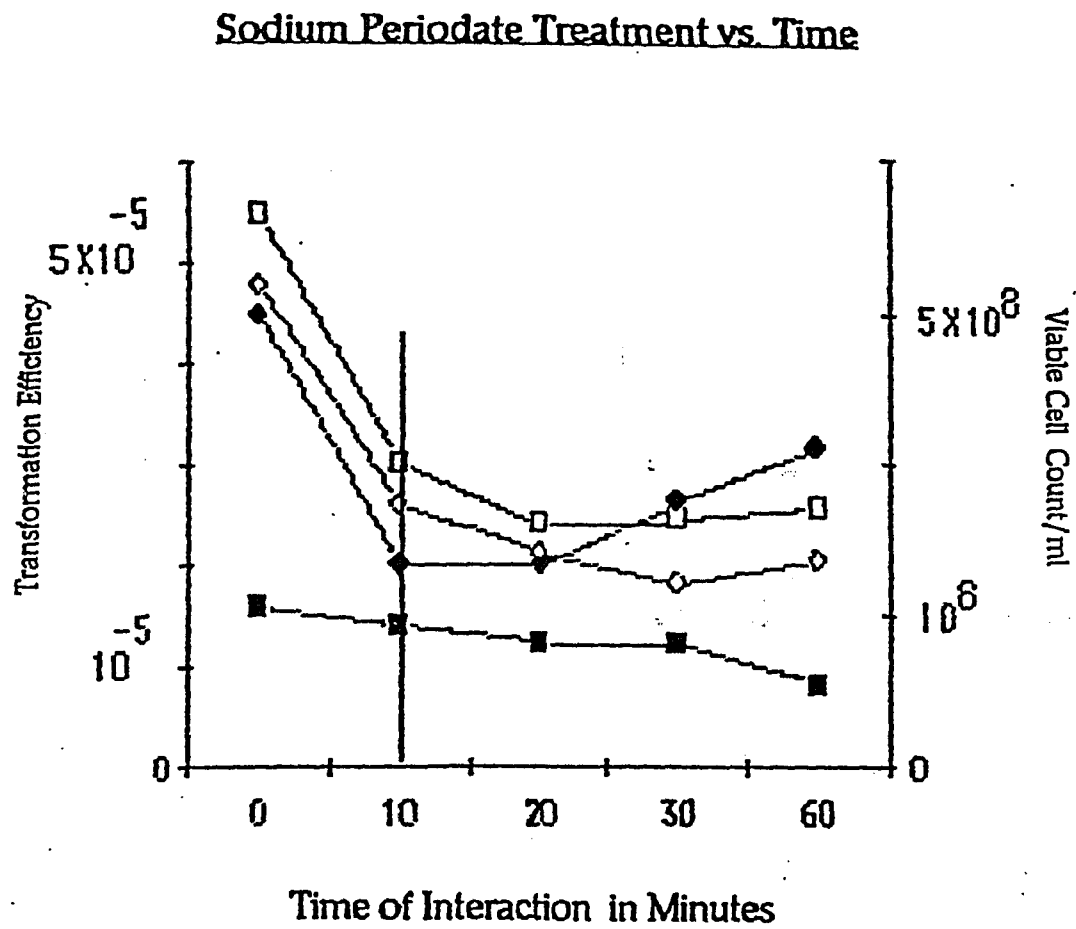
 Viable Counts for Transformation .

Phage λ Transfection Efficiencies .

 Phage λ Transfection Efficiencies .

 Viable Counts for Transfection .

Figure 8.



Both transformation and transfection were affected by the periodate treatment in a similar fashion in all strains tested. Pretreatment of plasmid DNA with periodate did not affect the transformation of recipients (data not shown). Similarly the treatment of the recipients with increasing amounts of NaIO_3 , the byproduct of NaIO_4 formed during the oxidative cleavage of carbohydrates containing vicinal alcoholic hydroxyl groups, did not affect plasmid transformation (data not shown).

The possibility that oxidation of the LPS at the OM might affect the configuration of some LPS-embedded protein receptors was examined by the exposure of periodate treated cells to intact phages. The efficiency of adsorption of intact bacteriophage $\phi 80$ vir. to periodate-treated cells was assayed by their plaque forming ability. No significant differences were seen in the number of plaques produced between the periodate treated (10^{-3} M), and the untreated cell controls (data not shown). This suggests that in *E. coli* the oxidation of the carbohydrate components of the LPS do not appear to produce radical changes in the OM architecture, which would alter the conformation of the $\phi 80$ receptor protein (KOB). The observed above effects of periodate on both transformation and transfection also suggest that the carbohydrate components of the LPS are not directly involved in the orientation of components at the DNA uptake sites.

Table 9

Effect of NaIO₄ on plasmid transformation in *E. coli*

Strain	[NaIO ₄]/0.2 ml	Transformation Efficiency	Viable Count
C600	0	7.44×10^{-6}	7.40×10^8
	10^{-4} M	7.00×10^{-6}	8.72×10^8
	5×10^{-4} M	7.30×10^{-6}	8.00×10^8
	10^{-3} M	5.40×10^{-6}	6.42×10^8
	3×10^{-3} M	$<10^{-8}$	$<10^6$
W3110	0	6.40×10^{-6}	5.30×10^8
	10^{-4} M	5.00×10^{-6}	3.21×10^8
	5×10^{-4} M	4.60×10^{-6}	7.00×10^8
	10^{-3} M	4.82×10^{-6}	3.63×10^8
	3×10^{-3} M	$<10^{-8}$	$<10^6$
SC401	0	8.40×10^{-6}	7.40×10^8
	10^{-4} M	8.85×10^{-6}	8.00×10^8
	5×10^{-4} M	10.20×10^{-6}	7.70×10^8
	10^{-3} M	3.00×10^{-6}	6.00×10^8
	3×10^{-3} M	$<10^{-8}$	$<10^6$

All strains in Table 9 were grown in LB and were transformed with the protocol of Davis et al 1980. Transformations were using 5 ng of pBR322 DNA after a 10 min periodate treatment. Transformation frequencies were calculated as the ratio of Amp^r colonies per ml to the total number of viable recipient cells per ml. Each experiment was the average of three independent trials.

Table 10
Effect of NaIO₄ on transfection in *E. coli*.

Strain	[NaIO ₄]/0.2 ml	Transfection Efficiency	Viable Count
C600	0	9.44×10^{-6}	8.72×10^8
	10^{-4} M	7.70×10^{-6}	11.00×10^8
	5×10^{-4} M	3.70×10^{-6}	11.00×10^8
	10^{-3} M	7.00×10^{-6}	7.00×10^8
	3×10^{-3} M	$<10^{-8}$	$<10^6$

Strain C600 was grown in LB and was transfected with the protocol of Davis et al., 1980. Transfections were done using 2 µg of λ vir. DNA after a 10 min. periodate treatment. Transfection frequencies were calculated as the ratio of plaques per ml to the total number of viable recipient cells per ml. C600 was used in all platings as indicator strain. The results are the average of three independent trials.

Effect of trypsin treatment on transformation.

The LPS oxidation experiments with periodate were instrumental in the further search of the nature of the molecules involved in DNA uptake during transformation. Several reports have suggested the possibility that transforming DNA binds initially to some OM protein (Taketo and Kuno, 1974; Weston et al., 1981; Hanahan, 1983). This possibility was further investigated here by assessing the effect of trypsin treatment on plasmid transformation in *E. coli*. Figure 4 shows a dose response curve of the effect of increasing concentrations of trypsin on transformation. Trypsin concentrations up to 5 µg/reaction mixture drastically reduced transformation without significantly affecting cell viability. The results on Table 11 show that treatment of intact *E. coli* with trypsin greatly reduced transformation with plasmid DNA. The apparent difference in transformation frequencies of trypsin treated recipients between the results in Figure 4 and Table 11, are due to a 10-fold dilution of the trypsin buffer used in the dose response experiments. The reduction in transformation frequencies of trypsin treated recipients was not due to loss of recipient cell viability, but because the enzyme probably removed some OM protein required for DNA binding, or uptake from the surface of recipient cells. This is in agreement with the previously reported observations, which showed that trypsin treatment of isolated cell membranes, which did not disrupt their organization greatly reduced the amount of labeled DNA bound to them (Weston et al., 1981).

Figure 4 . C600 was grown in LB , centrifuged for 5 min at 5,000 rpm at 4 ° C and was resuspended in saline . The cells were exposed to increasing amounts of trypsin at 37 ° C for 10 min . After the treatment the cells were removed by centrifugation and the pellets were resuspended into 50 mM CaCl₂ . The cells were induced for transformation by the protocol of Davis et al , (Davis et al . , 1980) . 15 ng of pBR322 DNA was used per 0.2 ml reaction mixture . Transformation frequencies were calculated as before . The results are the average of two trials .

Legend :



Plasmid pBR322 Transformation Frequency.



Viable Counts .

Figure 4

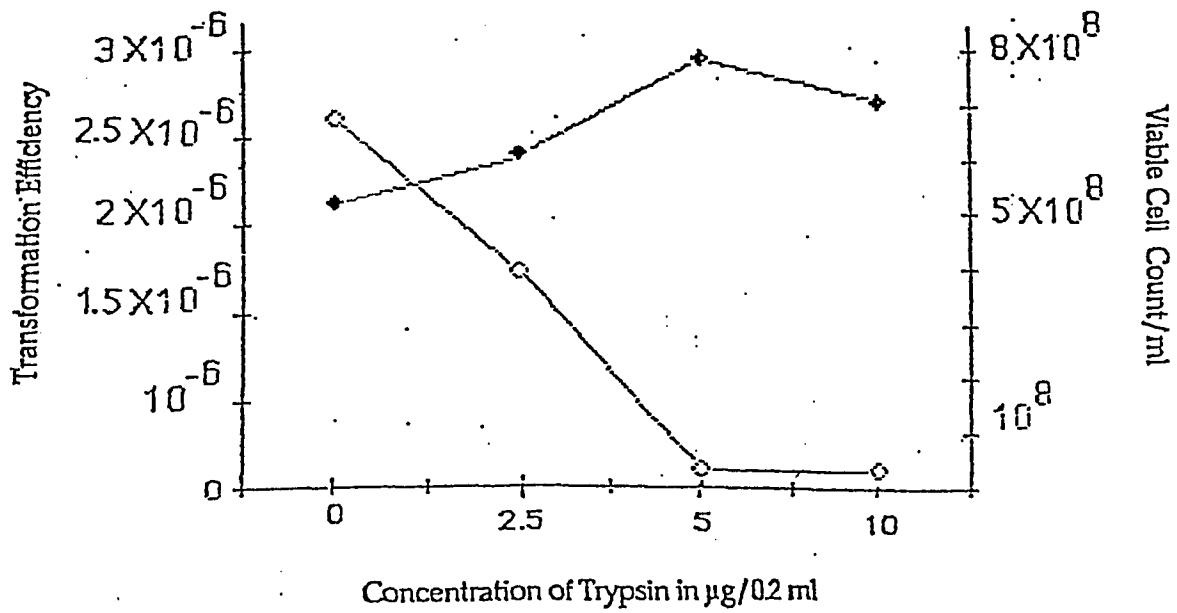
Concentration of Trypsin vs. Transformational Efficiency

Table 11

Effects of Trypsin treatment on Transformation in *E. coli*

Treatment	Transf. Efficiency x 10⁻⁶	Viable Count x 10⁸/ml
0.85% saline	2.65	8.60
Trypsin Buffer	1.10	5.20
Trypsin Buffer + Trypsin	0.22	6.01

Strain C600 was grown in LB and before the induction for transformation by the protocol of Davis et al. the cells were resuspended in saline, and exposed to 2.5 µg/0.2 ml trypsin for 10 min at 37°C. 15 ng of pBR322 DNA/0.2 ml reaction mixture was added to 0.1 ml of concentrated cells and the mixtures were chilled on ice for 10 min before the 90 sec heat shock at 42°C. Transformation and transfection efficiencies were calculated as previously described. The results are the average of three independent trials.

The results in Table 11 show that although transformation was reduced in trypsin buffer alone, probably because of the high EDTA content of the trypsin buffer, transformation frequency was greatly reduced by the action of trypsin. These results suggest that intact proteins of the outer membrane are needed for efficient plasmid uptake. The identity of the OM DNA binding protein is currently unknown. However the *btu* B protein, involved in vitamin B₁₂ uptake in *E. coli* have been implicated in this process (Hanahan, 1983).

Cytoplasmic Factors Affecting Transformation and Transfection.

Effect of vitamin B12 on transformation and transfection.

Hanahan reported a strong competition of vitamin B₁₂ with plasmid DNA for transformation and proposed that the *btu* B protein which is the vitamin B₁₂ uptake protein is involved in the uptake of plasmid DNA into *E. coli* (Hanahan, 1983). To replicate these observations and to further study the role of *btu* B protein in DNA uptake, several experiments involving the exposure of recipients to vitamin B₁₂ at different stages of transformation were performed. The presence of *btu* B protein on the surface of recipient cells was confirmed by the sensitivity of the cells to the *btu* B-specific phage BF23 (Dimiassi et al., 1973). The results in Table 12 show that relatively low

amounts (2×10^{-6} M) of vitamin B₁₂ during growth of cells, enhanced both transformation and transfection of *E. coli* C600 by about 50 %. However, the presence of 10-fold more B₁₂ (2×10^{-5} M), during the exposure of cells to DNA did not significantly alter the transformation, or transfection efficiencies of these cells. The results on Table 12 do not support Hanahan's observations (Hanahan, 1983). To ensure that this discrepancy was not a function of competence induction protocols, several strains of *E. coli* were assayed for plasmid transformation using the "high efficiency RbCl-organic solvent" protocol of Hanahan, in the presence and absence of vitamin B₁₂. The results of these experiments are shown on Table 13. Transformational frequencies obtained with the "high efficiency" protocol were not significantly affected by vitamin B₁₂. A dose response of the effect of increasing amounts of vitamin B₁₂ on plasmid transformation, showed that transformation efficiencies were unaffected even at very high levels of the vitamin (Figure 5).

Effects of osmolarity changes on transformation.

Increases in osmotic strength of bacterial culture media reportedly induce both qualitative and quantitative changes in the OM proteins of bacteria during adaptation to the media (Villarejo et al., 1986). An investigation was carried out to determine whether these changes in the OM protein content

affect competence . The requirement of partial plasmolysis of Ca^{2+} -treated cells to attain optimal transformation frequency has been previously proposed (Bukau et al . , 1985) . In the present study , the effects of osmotically induced changes , and osmoprotective compounds on the process of transformation were determined .

Table 12

Effect of vitamin B₁₂ on Transformation and Transfection in *E. coli*.

Growth Medium	Reaction Mixture	Transformation Efficiency	Viable Count /ml	Transfection Efficiency	Viable Count /ml
LB-B ₁₂	-B ₁₂	3.50X10 ⁻⁶	7.45X10 ⁸	3.04X10 ⁻⁶	7.05X10 ⁸
	+B ₁₂	2.82X10 ⁻⁶	7.12X10 ⁸	3.50X10 ⁻⁶	7.20X10 ⁸
LB+B ₁₂	-B ₁₂	7.10X10 ⁻⁶	6.72X10 ⁸	9.61X10 ⁻⁶	6.42X10 ⁸
	+B ₁₂	7.00X10 ⁻⁶	6.40X10 ⁸	8.41X10 ⁻⁶	6.42X10 ⁸

Strain C600 was grown in LB with or without 2×10^{-6} M cyanocobalamin . Cells were harvested and rendered competent by the protocol of Davis et al . (Davis et al . , 1980) . Competent cells (0.1 ml) were exposed to 2×10^{-5} M B₁₂ in TE buffer and 5 ng pBR322 and 200 ng λ vir DNA/0.2 ml reaction mixture . Transformation and transfection efficiencies were calculated as before . The results are the average of two trials .

Table 13

Effect of B₁₂ on the "high efficiency" transformation protocol¹

Strains	B ₁₂	Transformation Efficiency	Viable Count X10 ⁸ /ml
C600	-	2.10 X10 ⁻⁵	5.20
	+	2.75 X10 ⁻⁵	3.55
HB101	-	1.73 X10 ⁻⁵	5.00
	+	3.01 X10 ⁻⁵	3.00
SC401	-	7.02 X10 ⁻⁵	1.25
	+	3.40 X10 ⁻⁵	2.70

¹ The various strains were grown in LB , and were transformed by the protocol of Hanahan (Hanahan , 1983) using 5 ng/tube pCV35 DNA . Each DNA - cell reaction mixture contained 4X10⁻⁵ M cyanocobalamine . The results on Table 13 are the average of two trials .

Figure 5. C600 was induced for competence by the method of Davis et al., (Davis et al., 1980). The competent cells were exposed to increasing amounts of vitamin B12 in the cell-DNA reaction mixture for 10 min at 0°C. Each reaction mixture (0.2 ml) contained 15 ng of pBR322 DNA. Following a 90 sec heat shock treatment at 42°C, the transformants were phenotypically expressed, and plated on LB - ampicillin plates. The results in Figure 5 are the average of three trials.

Legend :

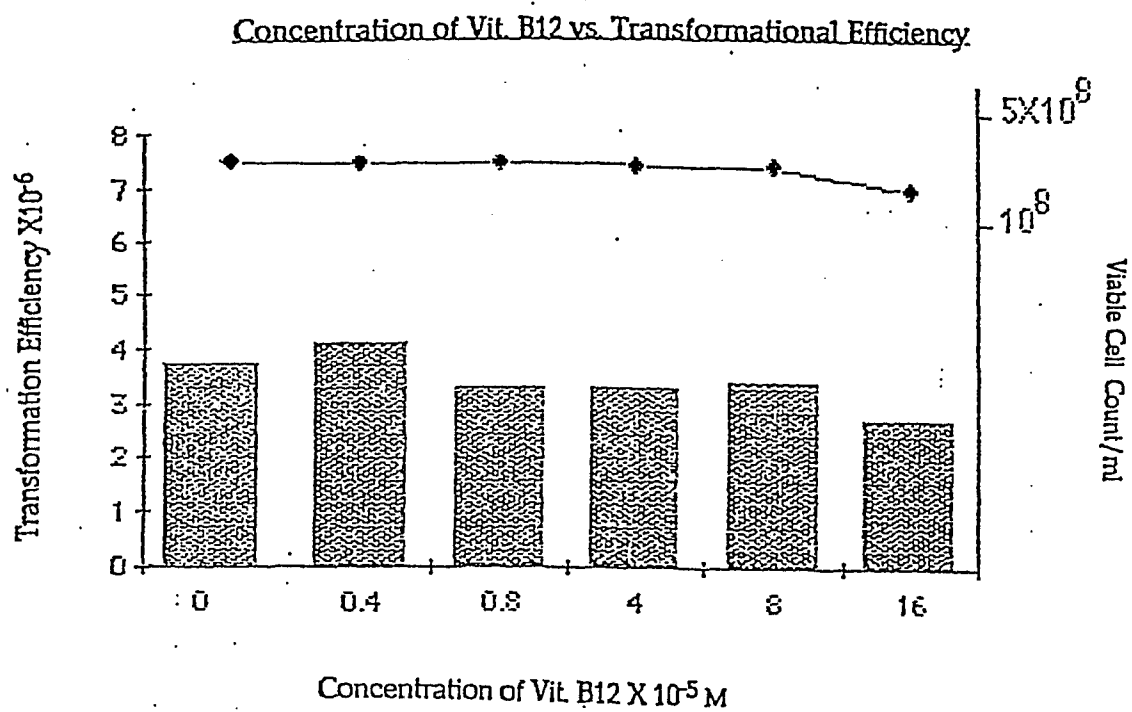


Plasmid pBR322 Transformation Frequency .



Viable Counts .

Figure 5



Concentrations of 0.3 M NaCl had little effect on the growth rates of cells grown in Penassay medium as shown in Figure 6 , but the cellular growth rate about doubled in the M63 medium , as seen in Figure 7. The addition of small amounts of known osmoprotectants proline and betaine to the salt-adjusted M63 media , restore the cellular growth rate to that of the osmotically unaltered control . The high level of NaCl in Penassay medium was probably unable to affect growth rates of cells because this medium contains enough proline to osmoprotect the cells .

The efficiencies of transformation of both osmotically stressed and osmoprotected cells are presented in Table 14 . Cells that were grown in either M63 , or Penassay media under conditions of osmotic stress , were not only significantly less transformable than the osmotically unstressed controls , but were also less viable . The supplementing of osmotically stressed cells with either proline or betaine in the M63 medium , resulted in partial restoration of cellular viability , and a slight enhancement in transformation frequencies . This effect was more pronounced with proline as osmoprotective than betaine . No such enhancement in transformation frequencies was observed in cells osmoprotected by either compound in Penassay broth .

Figure 6 . Strain W3110 was inoculated at 1:100 dilution into four 125 ml flasks each containing 20 ml of Difco Penassay broth . To three flasks , NaCl was added to a final concentration of 0.3 M^* , while the fourth flask was untreated , and served as control . Two of the NaCl treated flasks were also supplemented with 10^{-3} M proline and $5 \times 10^{-4} \text{ M}$ glycine betaine respectively . The cultures were grown aerobically at 37° C . Cell growth was monitored by increases in optical density measured in a Klett-Summerson colorimeter at 590 nm . The results in Figure 6 are the average of three trials .

Legend :



Penassay Control (no NaCl) .



Penassay with 0.3 M NaCl .



Penassay with 0.3 M NaCl and proline .



Penassay with 0.3 M NaCl and glycine-betaine.

*Penassay may contain some additional NaCl .

Figure 6

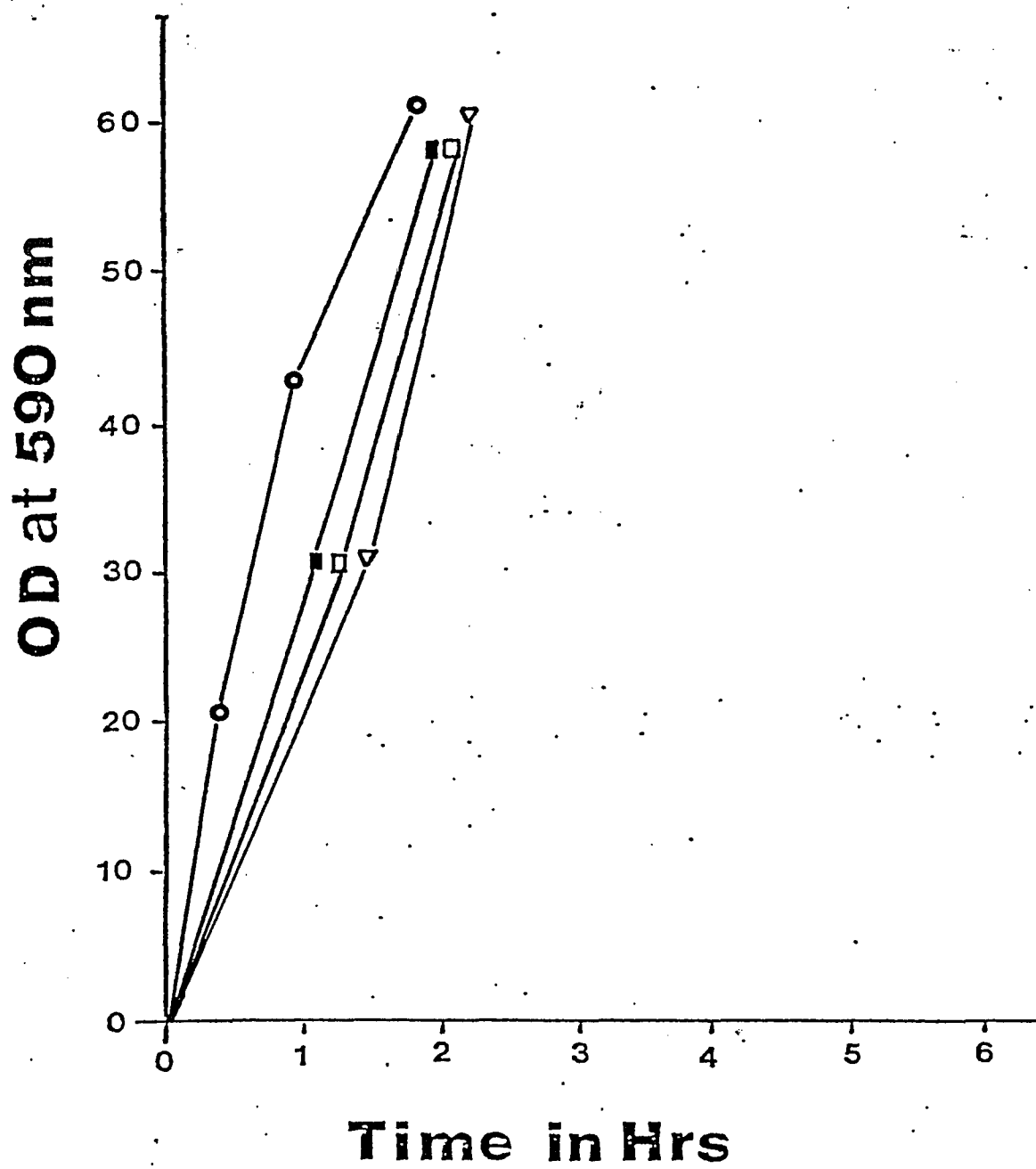


Figure 7. Strain W3110 was inoculated at 1:100 dilution into four 125 ml flasks each containing 20 ml of M63 medium, supplemented with 1 $\mu\text{g/ml}$ thiamine and 0.2% glucose. To three flasks NaCl was added to a final concentration of 0.3 M, while the fourth flask was untreated, and served as control. Two of the NaCl treated flasks were further supplemented with 10^{-3} M proline and 5×10^{-4} M glycine-betaine respectively. The cultures were grown aerobically at 37°C. Cell growth was monitored by increases in optical density measured in a Klett-Summerson colorimeter at 590 nm. The results in Figure 7 are the average of three trials.

Legend :

- 
Control M63 medium (no NaCl).
 Control M63 medium (no NaCl).
- 
M63 medium with 0.3 M NaCl.
- 
M63 medium with 0.3 M NaCl and proline.
- 
M63 medium with 0.3 M NaCl and glycine-betaine

Figure 7

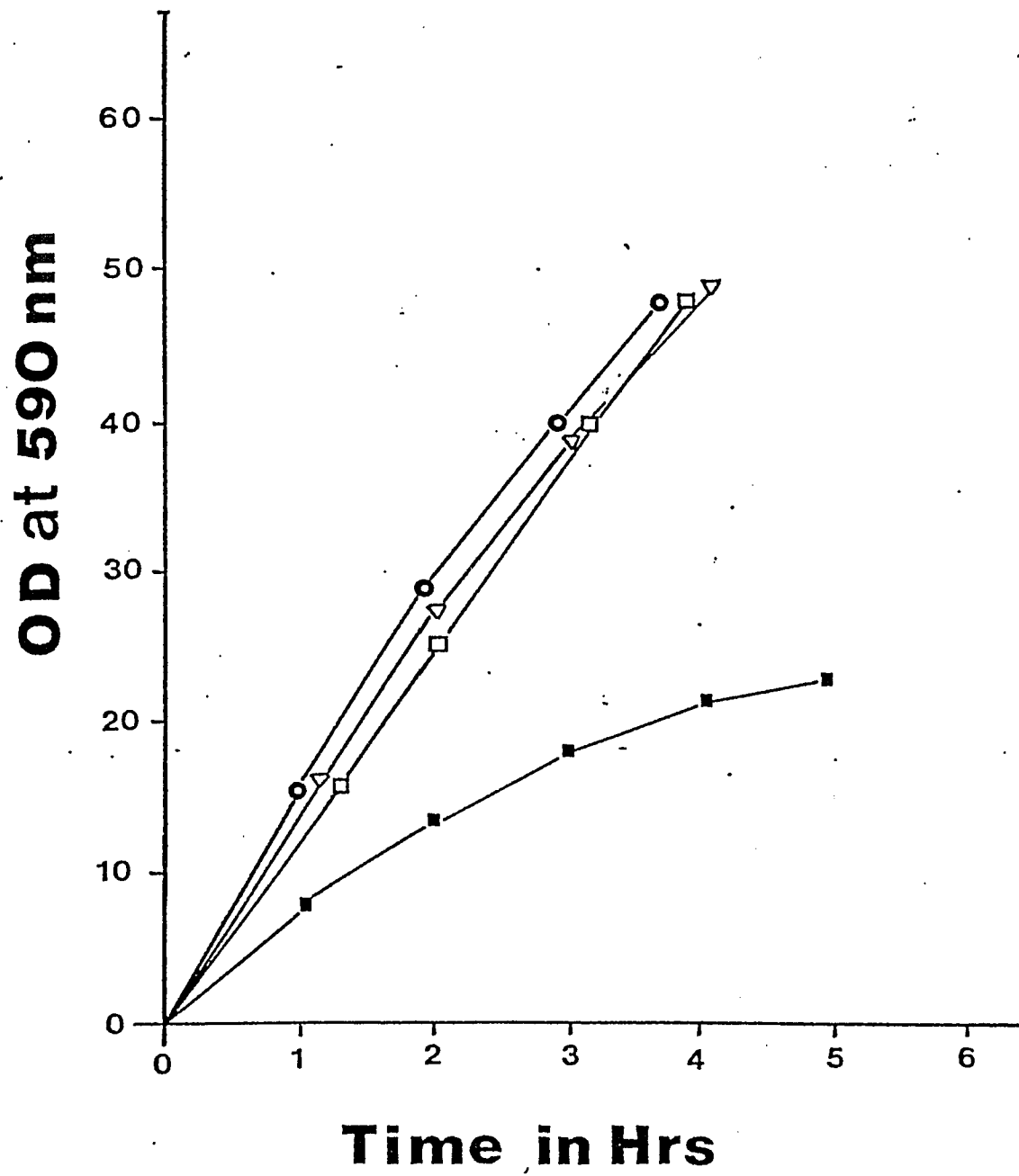


Table 14**Effect of Osmotic Stress and Osmoprotection on Transformation**

Growth Medium	Added Reagents	Transformation Efficiency $\times 10^{-6}$	Viable Count $\times 10^8$/ ml
M63 ¹	None	1.55	6.10
	NaCl	1.31	1.70
	NaCl + Pro.	6.21	4.80
	NaCl + Bet.	2.02	4.80
Penassay	None	4.20	10.32
	NaCl	1.10	7.80
	NaCl + Pro.	1.31	6.81
	NaCl + Bet.	1.17	6.00

¹M63 was supplemented with 0.2 % glucose and 1 μ g/ ml B₁ . Strain W3110 was grown in M63 and Difco Penassay broth . The cells then were transformed or transfected with 5 ng pBR322 DNA . Transformants were selected on LB-ampicillin (50 μ g/ml) plates , while transfectants were selected on TB plates using soft TB overlay supplemented with 10mg/m MgSO₄ , and C600 as indicator strain . Viable counts were diluted in LB broth , and plated on LB plates usually at 10⁻⁶ dilutions . The concentrations of NaCl , proline , and betaine were 0.3 M , 0.001 M , and 0.0005 M respectively. The results in Table 14 are the average of three trials.

Effect of amino acids on transformation and transfection.

Although competence in *E. coli* is artificially induced, recent findings by Reusch et al. have suggested that competence in this organism might be to some degree physiologically regulated (Reusch et al., 1986). The possibility that certain growth factors in the culture media of *E. coli* could enhance its transformability was tested. Mixtures of various amino acids, that reportedly affected transformation in *B. subtilis* (Erickson, 1970), were tested in the *E. coli* transformation system.

Strains SC401 and C600 that usually transformed most efficiently, were aerobically grown in defined minimal medium (SSA), supplemented with mixtures of amino acids that were either stimulatory or inhibitory to *B. subtilis*. These strains were also grown in minimal medium in the presence of a commercial casein hydrolysate. All cultures were transformed with plasmid pBR322 DNA, using the standard cold CaCl_2 protocol (Davis et al., 1980). The results in Table 15 seem to support the idea of some physiological regulation of competence in *E. coli*. This is particularly evident in recipients grown with casamino acids, where despite the obtained high cell densities, only a small number of cells transform. Apparently some component of the casamino acid solution is inhibitory to transformation in *E. coli*. This effect is similar to the inhibitory effect that casein hydrolysates exert on *B. subtilis* transformation (Erickson, 1970). The amino acids that are stimulatory to *B. subtilis*, arginine, histidine, threonine, glycine, lysine, and methionine

enhanced the transformation frequencies of C600 more than two-fold , but had little effect on strain SC401 . On the other hand , the amino acids inhibitory to *E. subtilis* , alanine , glutamic acid , proline , leucine , and isoleucine , somehow prevented the development of competence in both C600 and SC401 . The growth of recipients in the presence of amino acid arginine alone , did not result in more efficient transformation (data not shown) . This is contrast to *E. subtilis* where this amino acid is very effective in stimulating transformation (Dr Rudner personal communication) .

The mechanism by which externally supplied amino acids affect transformation in *E. coli* is unknown . However , the results in Table 15 suggest that growth on the various amino acid mixtures affects the ability of recipient cells to become competent differently . The extent of these amino acid effects vary , between the two different strains tested , and must be the result of some cytoplasmic function , since these effects are not the result of a direct enhancement , or prevention of interaction of DNA with recipient cells

Table 15
Effect of amino acids on plasmid transformation of *E. coli*

	<u>C600</u>		<u>SC401</u>	
Growth	Transformation	Viable	Transformation	Viable
Medium	Efficiency X10⁻⁶	Count X 10⁸/ml	Efficiency X 10⁻⁵	Count X 10⁸/ml
Min + Requir.	2.40	4.54	14.60	3.00
Min + Stim.aa	5.45	4.50	11.40	5.00
Min + Inhib.aa	0.70	3.81	7.10	6.10
Min + Cas. aa	0.07	7.60	3.30	7.80

C600 was grown in SSA supplemented with leucine , threonine, glucose and B₁ , while SC401 was grown in SSA supplemented with tryptophane , glucose, and B₁ . Each strain was also grown in the above media supplemented with inhibitory , stimulatory , or caseamino acids at a final concentration of 40 µg/ml . Stimulatory amino acids are : Arginine , Histidine Glycine , Methionine , Lysine , and Aspartic acid . Inhibitory amino acids are : Alanine , Leucine , Threonine , Proline . The cultures were grown transformed with the protocol of Davis et al . , 1980 . Transformation was carried out using 5 ng of pBR322 DNA / 200 µl reaction mixture and transformation frequencies were calculated as before . The results are the average of three trials .

Effect of Antibiotics on Transformation and Transfection .

In order to assess the role of cellular proteins in genetic transformation of *E. coli* , several experiments were performed involving the chemical alterations of the cellular metabolism . In these experiments four different antibiotics were employed , each with a different site of action in the cell . The bacteriostatic antibiotics chloramphenicol (Cm) and tetracycline (Tet) both of which block protein synthesis in bacteria in a reversible manner , were used to experimentally modulate cytoplasmic protein synthesis during the different phases of competence induction . While the antibiotics 5-diazouracil (Dzu) and nalidixic acid (Nal) were employed to measure the effects of the inhibition of bacterial cell division and DNA synthesis on *E. coli* transformation and transfection . The addition of Cm , Tet , Dzu , and Nal , to actively growing cultures of *E. coli* resulted in rapid cessation of growth . The extent of growth inhibition was dependent on the particular antibiotic used . The results in Table 16 show that parallel with this growth inhibition , were marked reductions in the transfection and transformation efficiencies in the Dzu and Nal-treated cells . However , no such reductions were observed in Cm and Tet-treated recipients . The finding that Cm does not affect the efficiency of plasmid transformation in *E. coli* has been previously reported by Reush et al . (Reush et al . , 1986) . In this study , the efficiencies of transformation and transfection were also monitored after a "recovery" period of up to two cell duplications following the removal of the antibiotics . Growth of the

cells after the removal of the antibiotics is shown in Figure 8. The results on Table 16 show that recovery of recipient *E. coli* from the exposure to Dzu and NaI did not significantly affect their transformabilities, but recovery from treatment with Cm and Tet enhanced plasmid transformation and phage transfection efficiencies 4 to 10-fold. The general nature of this phenomenon is evident from the results in Table 17, which shows that this enhancement occurs in all *E. coli* strains irrespective of their genotype, and with topologically different DNA molecules.

These increases in transformational and transfectional efficiencies were transient, since after one hour of recovery transformational efficiencies declined, but they did not reach those of untreated controls (Figure 9). Also a similar, although less dramatic response has been observed with tetracycline (data not shown). Among the possible reasons for enhancement of transformation and transfection by Cm in early recovering cells is either increased envelope permeability to DNA, or more efficient establishment of incoming DNA. These possibilities were investigated in this study.

E. coli is usually resistant for the antibiotic actinomycin-D because the drug cannot penetrate the cell envelope. Recipients recovering from Cm treatment were tested for sensitivity to this drug at concentrations up to 30 µg/ml. None of the tested recipients was sensitive to this drug. This served as an indication that these cells were not abnormally permeable to outside macromolecules.

Figure 8. Strain C600 was grown in LB broth until a density corresponding to an absorbance on the Klett-Summerson colorimeter of about 40 at 590 nm. The culture was divided into four subcultures, three of which were treated with three different antibiotics, while the fourth culture was untreated and served as control. The concentrations of the various antibiotics are described in Table 16. The cultures were incubated for 30 min at 37 °C and were subsequently pelleted by centrifugation and washed twice with equal volume prewarmed LB. After the washings, the cells were centrifuged, and the pellets were resuspended into equal volumes of fresh LB, and grown for an additional hour at 37 °C. The results of Figure 8 are the average of three trials.

Legend:



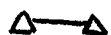
Untreated Cell Control.



Growth of Chloramphenicol Treated Cells

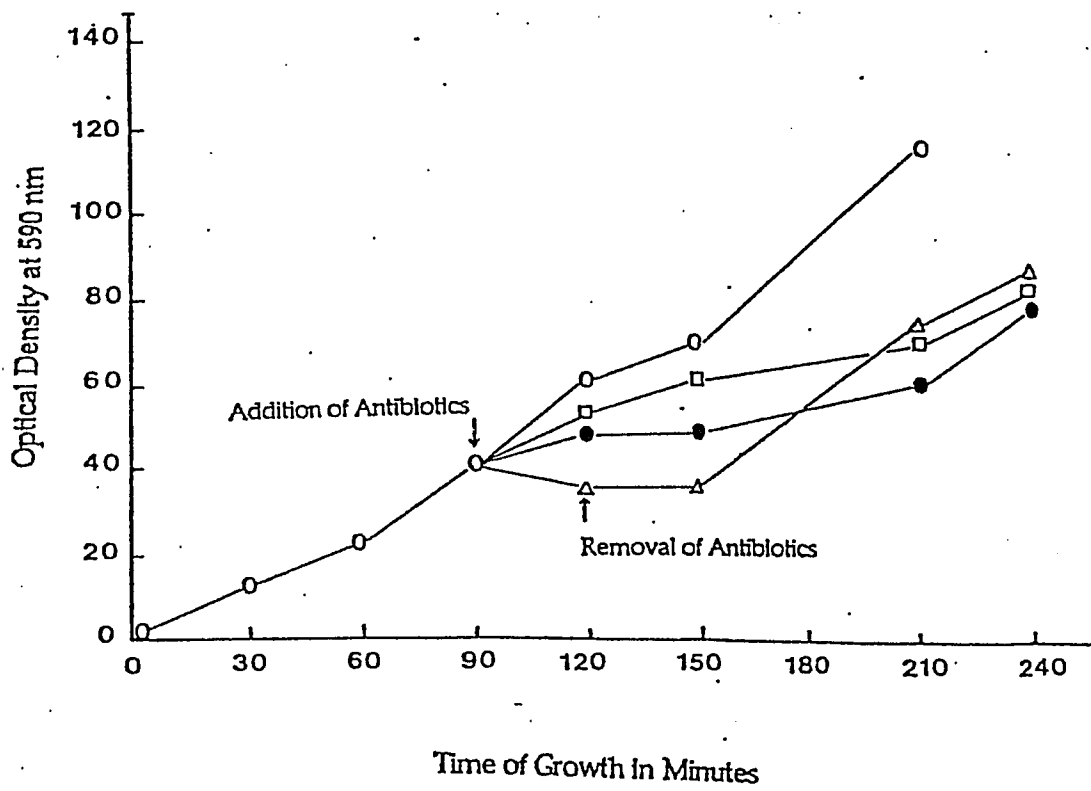


Growth of Diazo-Uracil Treated Cells.



Growth of Tetracycline Treated Cells.

Figure 8.

Cell Growth of *E. coli* vs. Antibiotic Treatments

Confirmation of these results was obtained by radiolabeled plasmid pBR322 uptake studies . These showed that recipient cells in recovery did not bind , or internalize significantly more DNA than those of the untreated control (Table 18) .

Since the observed increases in transformation and transfection of recovering cells are not due to enhanced uptake of DNA , perhaps more cells are able to successfully establish DNA after its uptake . The conditions required for the establishment of DNA in recipient cells are unknown . The results in Table 18 suggests that recovering cells of C600 synthesize about 50% more protein than their non-recovering counterparts . Presently it is unclear how such an increase in protein synthesis could be responsible for the observed 10-fold increase in the efficiencies of transformation and transfection .

Table 16

Effects of Antibiotics on Transformation and Transfection of *E. coli*

Antibiotic	Treatment	Transformation Efficiency $\times 10^{-6}$	Transfection Efficiency $\times 10^{-6}$	Viable Counts $\times 10^8/\text{ml}$
Chloramphenicol (100 $\mu\text{g}/\text{ml}$)	NONE	8.20	12.10	6.00
	BEFORE	6.03	9.10	3.00
	AFTER	38.00	130.00	3.30
Tetracycline. (15 $\mu\text{g}/\text{ml}$)	NONE	3.44	3.50	4.50
	BEFORE	4.73	3.00	1.60
	AFTER	12.00	25.00	4.30
5- Diazouracil (2.5 $\mu\text{g}/\text{ml}$)	NONE	18.51	21.55	5.40
	BEFORE	5.00	12.30	3.52
	AFTER	8.60	14.60	2.81
Nalidixic acid (10.0 $\mu\text{g}/\text{ml}$)	NONE	12.80	17.61	6.42
	BEFORE	5.80	6.53	3.00
	AFTER	6.70	12.45	3.20

Strain C600 was exposed to various antibiotics for 30 min at the concentrations indicated in Table 16 at 37° C in LB broth . Following the above exposure to the antibiotics , cells were washed with LB and were allowed to recover in fresh LB . The samples of cells that were washed , but were not allowed to recover before were transformed were designated BEFORE while cells that recovered for 30 min at 37° C were designated AFTER . Transformations and transfections were performed with 5 ng pBR322 and 200 ng λ vir DNA/ 0.2 ml of reaction mixtures . The transformation and transfection efficiencies were obtained as before and each value in Table 16 is the average of three experiments .

Table 17

Effects of Chloramphenicol on Transformation and Transfection

Transformation and				
<u>Strain</u>	<u>Treatment</u>	<u>DNA</u>	<u>Transfection Efficiencies</u>	<u>Viable Count/ml</u>
C600	NONE	pBR322	8.20×10^{-6}	6.00×10^8
		λ vir.	12.10×10^{-6}	
	BEFORE	pBR322	6.03×10^{-6}	3.00×10^8
	RECOVERY	λ vir.	9.10×10^{-6}	
	AFTER	pBR322	38.00×10^{-6}	3.30×10^8
	RECOVERY	λ vir.	130.00×10^{-6}	
SC401	NONE	pBR322	11.00×10^{-6}	6.00×10^8
		λ vir.	26.61×10^{-6}	
	BEFORE	pBR322	5.00×10^{-6}	3.00×10^8
	RECOVERY	λ vir.	23.20×10^{-6}	
	AFTER	pBR322	24.50×10^{-6}	5.65×10^8
	RECOVERY	λ vir.	143.00×10^{-6}	

(continued on next page)

Table 17(continued)**Effects of Chloramphenicol on Transformation and Transfection**

Transformation and				
Strain	Treatment	DNA	Transfection Efficiencies	Viable Count/ml
AB1157	NONE	pBR322	10.50×10^{-6}	2.53×10^8
		λ vir.	3.84×10^{-6}	
		T7	0.00	
	BEFORE	pBR322	6.20×10^{-6}	1.25×10^8
	RECOVERY	λ vir.	12.00×10^{-6}	
		T7	0.20×10^{-6}	
	AFTER	pBR322	48.00×10^{-6}	2.21×10^8
	RECOVERY	λ vir.	87.50×10^{-6}	
		T7	0.60×10^{-6}	
JC7623	NONE	pBR322	10.21×10^{-6}	1.50×10^8
		λ vir.	35.60×10^{-6}	
		T7	4.05×10^{-6}	
	BEFORE	pBR322	0.91×10^{-6}	1.50×10^8
	RECOVERY	λ vir.	5.50×10^{-6}	
		T7	1.10×10^{-6}	
	AFTER	pBR322	11.80×10^{-6}	2.00×10^8
	RECOVERY	λ vir.	73.00×10^{-6}	

Table 17(continued)

Effects of Chloramphenicol on Transformation and Transfection

		Transformation and		
<u>Strain</u>	<u>Treatment</u>	<u>DNA</u>	<u>Transfection Efficiencies</u>	<u>Viable Count/ml</u>
JC7623	AFTER RECOVERY	T7	9.25×10^{-6}	2.00×10^8

The various *E. coli* strains were exposed to 100 µg/ml chloramphenicol for 30 min at 37°C in LB broth. The cells were washed with fresh LB and half of them were allowed to recover in LB for one doupplication. The samples were transformed and transfected with 15 ng pBR322 and 600 ng λ vir DNA, and 260 µg of T7 DNA/ 0.2 ml of reaction mixture. The transformation and transfection efficiencies were obtained as before. Each value in Table 17 is the average of three experiments.

Figure 9 . Strain AB1157 was grown in LB broth until a density corresponding to an absorbance on the Klett-Summerson colorimeter of about 40 at 590 nm . The culture was divided into four subcultures , three of which were treated with 0.10 mg/ml chloramphenicol while the fourth culture was untreated and served as control . The cultures were incubated for 30 min at 37 °C , and were subsequently pelleted by centrifugation and washed twice with equal volume prewarmed LB . After the washings , the cells were centrifuged , and the pellets were resuspended into equal volumes of fresh LB . One of the washed cultures was induced for competence , while the other two were further grown at 37 °C to densities corresponding to absorbances on the Klett-Summerson colorimeter of about 60 and 90 respectively . All cultures were induced for competence by the method of Davis et al , (Davis et al , . 1980)

Transformations and transfections employed 5 ng of pBR322 DNA and 200ng of λ per 0.2 ml of reaction mixture . Transformation and transfection frequencies were measured as before . The results in Figure 9 are the average of three trials ,

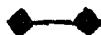
Legend :



Plasmid pBR322 Transformation Frequency .



Phage λ Transfection Frequency .



Viable Counts .

Figure 9.

Transformational/Transfectional Efficiencies of *E. coli*
vs. Chloramphenicol Treatment

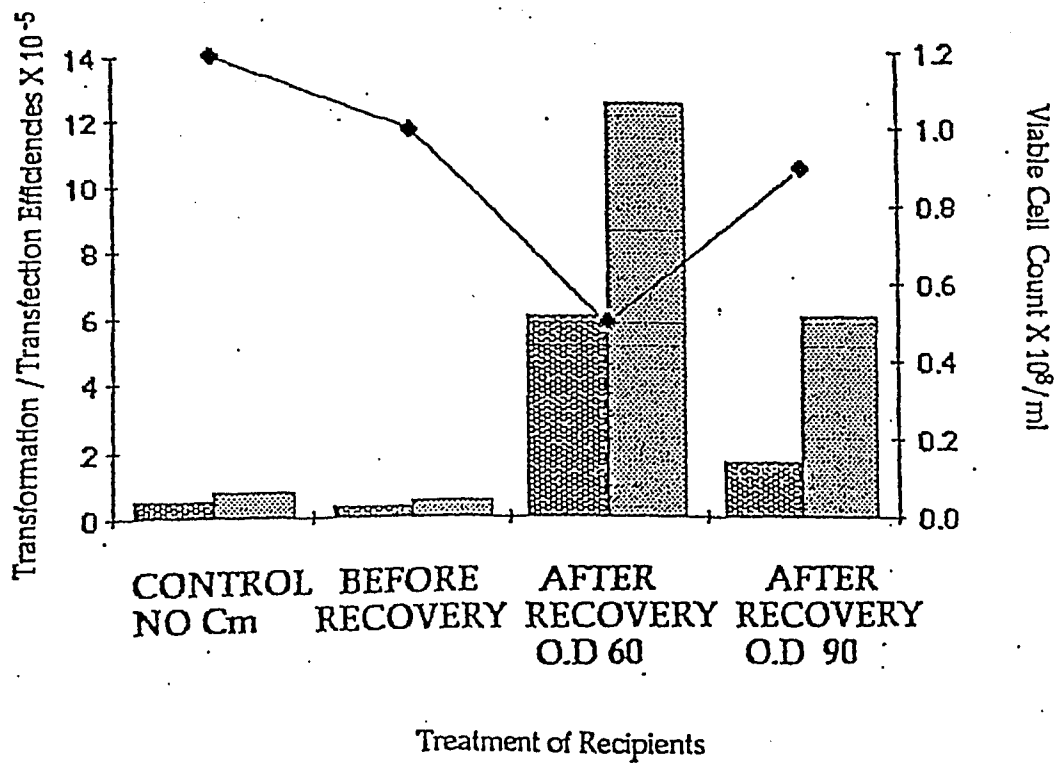


Figure 10 . Strain SC403 was grown in LB broth until a density corresponding to an absorbance on the Klett-Summerson colorimeter of about 40 at 590 nm . The culture was centrifuged and the pellet washed in prewarmed SSA . After a second centrifugation the cell were resuspended into equal volume of SSA supplemented with requirements and glucose but not uracil . The culture was divided into six subcultures , three of which were supplemented with 40 µg /ml uracil , while the other three were not . The downshifted cultures were allowed to acclimate to the minimal medium for 30 min at 37 °C , and two cultures with and two without uracil were treated with 0.10 mg/ml chloramphenicol , while the last two cultures were untreated and served as control . The cultures were incubated for 30 min at 37 °C and were subsequently pelleted by centrifugation . The pellets were resuspended into equal volumes of fresh LB and were either induced for competence , or were further grown at 37 °C to a density corresponding to an absorbance on the Klett-Summerson colorimeter of about 60 at 590 nm . All cultures were induced for competence by the method of Davis et al (Davis et al , . 1980) . Transformation and transfection frequencies were measured as before . The results in Figure 9 are the average of three trials .

Legend :



Plasmid pBR322 Transformation Frequency with uracil .



Plasmid pBR322 Transformation Frequency without uracil .



Phage λ Transfection Frequency with uracil .



Phage λ Transfection Frequency without uracil .



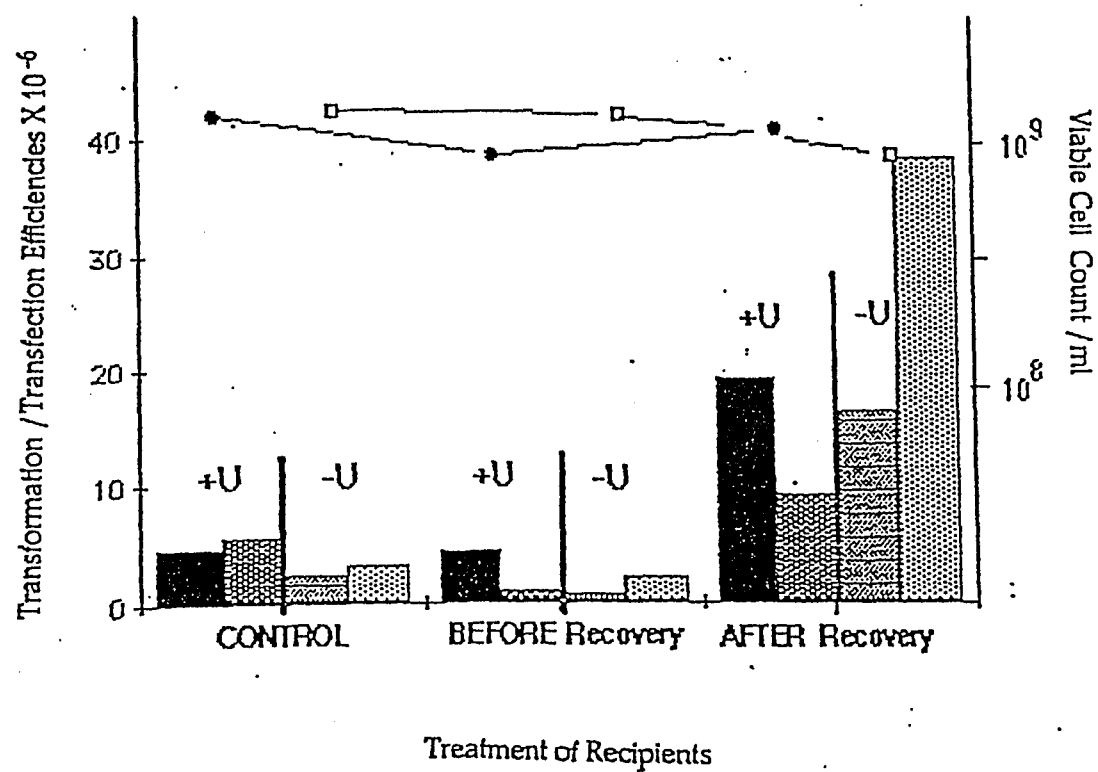
Viable Counts with uracil .



Viable Counts without uracil .

Figure 10

Transformation and Transfection Efficiencies in the Presence and
Absence of RNA Synthesis



Since RNA synthesis does not stop after Cm treatment, it is possible that the effects seen on transformation and transfection after Cm treatment are due to the presence of increased amounts of cytoplasmic RNA. This possibility was investigated by allowing auxotrophs of RNA synthesis to recover from Cm treatment, and assaying their transformation and transfection efficiencies. The results of transformation of an *E. coli* (SC403) which harbors a mutant *purF* allele is presented in Figure 10. Although cells of SC403 do not synthesize RNA in the absence of uracil, their transformation and transfection efficiencies were not diminished, in fact they were somewhat enhanced in the absence of RNA synthesis. This experiment clearly shows that RNA build up in the cytoplasm during the cessation of protein synthesis cannot account for the transformational enhancement of recipients recovering from inhibition of protein synthesis.

One possible explanation of why cells recovering from inhibition of protein synthesis have enhanced transformation capability is that these cells may be deficient in mechanisms that degrade donor DNA. Table 17 shows that the transformation and transfection efficiencies of two isogenic strains AB1157 (Rec+) and JC7623 (RecF), which differ from each other with respect to the presence of ExoV were not significantly different for plasmid DNA, but differed considerably in transformation by T7, a phage whose DNA is linear. During and after recovery, transformation with T7 DNA is observed, therefore there must be some inactivation of ExoV. The lack of increased amounts of

TCA-precipitable DNA fragments in the cytoplasm of transformed AB1157 with pBR322 during recovery from Cm treatment (Table 18) , suggests that differences in the amounts of cytoplasmic nucleases between the various stages of Cm treatment cannot alone account for the observed transformational enhancement during recovery .

Table 18

Effects of chloramphenicol treatment on ^3H DNA and ^{14}C leucine uptake

	<u>^3H-DNA</u>		<u>^{14}C Leucine</u>	
	Total cpm.	DNase Resistant cpm.	TCA ppt. cpm.	TCA ppt. cpm.
CONTROL	2324.60	330.60	293.30	395.00
BEFORE RECOVERY	2000.20	250.13	317.04	292.30
AFTER RECOVERY	2849.00	305.75	276.11	667.00

	<u>Transformation Frequency $\times 10^{-6}$</u>	<u>Viable Counts $\times 10^8/\text{ml}$</u>
CONTROL	2.21	8.50
BEFORE RECOVERY	5.20	2.50
AFTER RECOVERY	20.00	6.12

C600 was grown in LB broth and was induced for competence by the protocol of Davis et al ., 1980. 5ng (methyl-3H) Thymidine-labeled pBR322 DNA was added per 0.2 ml of reaction mixture. The amounts of total DNA, DNase resistant, and cytoplasmic DNA was determined as described in Methods and Materials. The uptake of labeled leucine was measured by adding 10 μ l of ^{14} C leucine of specific activity 54.4 mCi/ mM to pBR322 transformed recipients during phenotypic expression. The measurement of radioactive protein was described in Methods. Each DNA uptake, and leucine uptake was performed twice and was measured in duplicate and was standardized for normal background. Each sample was also tested for unlabeled pBR322 transformation. Frequency of transformation was calculated as previously described.

Effects of polyamines on transformation and transfection .

Polyamines enhance both the transformation of *E. subtilis* and the transfection of *E. coli* spheroplasts (Clark and Leach , 1980 ; Benzinger et al . 1971) . These compounds are able to form tight electrostatic bonds with polyanionic molecules like DNA , perhaps facilitating their effective precipitation on the cell surface . Studies were carried out to determine their effect on plasmid transformation and phage transfection using intact recipient cells .

Competent cells of strain C600 were treated with increasing concentrations of polyamines spermidine and putrescine on ice , and were subsequently assayed for transformability with plasmid pBR322 DNA , as described in Methods . Alternately , plasmid DNA was pretreated with the polyamines on ice and was added to competent recipient cells . Table 19 shows that plasmid transformation in either mode of treatment was reduced as a function of polyamine concentration , while recipient viability remained more or less unaffected . The reduction in transformation efficiency was most pronounced when the transforming DNA was pre-treated with the polyamines prior to transformation . This suggests that the polyamines affect transformation mainly by interacting with DNA , and to a lesser degree with recipient cells . The tight interaction of the positively charged polyamines with

DNA has been well documented (Ames and Dubin , 1960 ; Felsenfeld and Huang , 1960) . DNA molecules may aggregate and even precipitate at high enough concentrations of polyamines . In the presence of 10^{-5} M spermidine approximately 93% of the transfection efficiency was lost , with only 50% loss of viability . While at 5×10^{-5} M concentration spermidine was lethal to the cells .

Of the two naturally occurring polyamines , the more efficient in inhibiting transformation was putrescine , which at 10^{-5} M concentration showed transformation efficiency of about 9% of the control . Similar results were also obtained in SC401 transfections with phage DNA . Table 20 shows that increasing amounts of spermidine reduced transfection efficiencies without affecting viability of the recipients .

Table 19

Effects of Polyamines on Plasmid Transformation of *E. coli*

Polyamine	Component Affected ¹	Concentration of Polyamine	Transformation Efficiency $\times 10^{-5}$	Viable Count $\times 10^8/\text{ml}$
Spermidine	DNA	0	2.94	2.80
		$2 \times 10^{-7} \text{ M}$	2.00	2.63
		$2 \times 10^{-6} \text{ M}$	1.30	2.60
		$2 \times 10^{-5} \text{ M}$	0.73	2.11
		$2 \times 10^{-4} \text{ M}$	0.23	2.30
	Cells	0	1.02	4.75
		$2 \times 10^{-7} \text{ M}$	1.34	4.33
		$2 \times 10^{-6} \text{ M}$	0.72	4.20
		$2 \times 10^{-5} \text{ M}$	0.30	4.62
		$2 \times 10^{-4} \text{ M}$	0.33	3.14
	Cells+DNA	0	2.01	2.85
		$2 \times 10^{-5} \text{ M}$	1.12	2.40
		$2 \times 10^{-4} \text{ M}$	0.72	2.35
Putrescine	DNA	0	1.20	2.50
		10^{-6} M	0.20	1.54
		10^{-5} M	0.24	1.27
		10^{-4} M	$>10^{-8}$	0.01
	Cells	0	1.10	2.35
		10^{-6} M	0.25	1.83
		10^{-5} M	0.10	2.00
		10^{-4} M	$>10^{-8}$	0.03

¹ 10 ng of plasmid pBR322 DNA was either preincubated on ice for 10 min with polyamines before it was added to competent cells of *E. coli* C600, or the competent cells themselves, were exposed to the polyamines for 10 min on ice before they were added to 10 ng of plasmid pBR322 DNA, or the polyamines were added to the cell-DNA mixture. Transformation efficiencies were calculated as before. The results in Table 19 are the average of three trials.

Table 20

Effects of Spermidine on Donor DNA in Transfection

Spermidine Concentration ¹	Transfection Efficiency X 10 ⁻⁶	Viable Count X 10 ⁸ /ml
0	3.41	6.00
10 ⁻⁷ M	2.00	5.50
10 ⁻⁶ M	2.50	6.72
10 ⁻⁵ M	0.23	2.80
5X10 ⁻⁵ M	< 0.01	0.01

¹Spermidine concentration contained in 0.2 ml of reaction mixture. Phage ϕ 80 DNA had a concentration of 100 ng / 0.2 ml reaction mixture. DNA and spermidine were allowed to interact on ice for thirty minutes followed by the addition of competent SC401 cells. Transfectants were selected on TB plates with C600 as indicator strain. Transfection efficiency was calculated as above. The results are in Table 20 are the average of three experiments.

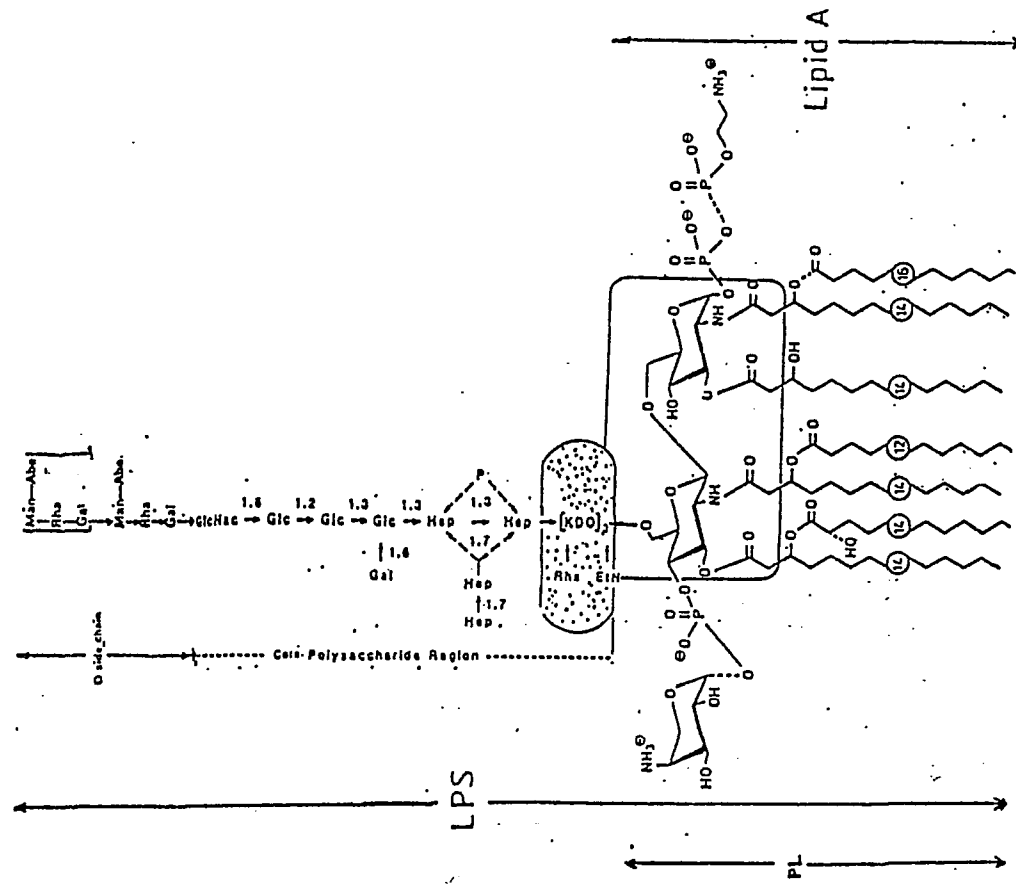
Figure 11 . Tentative structural view of the outer membrane lipopolysaccharide (LPS) of *E. coli* composed from studies of Coleman and Lelve, 1979 and Labischinski et al . 1985 .

Legend :

Glc = glucose , Gal = galactose , Rha = rhamnose , Man = manose ,
Abe = abequose , Et N = ethonolamine , KDO = 2 keto-3 -deoxyoctulonate .

PS = polysaccharide , LA = lipid A , PL = phospholidid ,
HR = hydrophilic region , LR = hydrophobic region .

Figure 11



DISCUSSION

In the present study several aspects of transformation and transfection in *E. coli* were investigated. A comparison of transformation efficiencies of several unrelated bacterial strains and three different transformation protocols showed that although certain strains of *E. coli* transformed under all tested protocols more efficiently than others, no apparent correlation could be drawn between transformability and cellular genotype. Such lack of correlation between various cellular genotypes and transformation has been, with the exception of galactose-epimerase (*gal*⁻ E) mutants (vanDie et al., 1983), also reported by Hanahan (Hanahan, 1983). The lack of a direct link between enhanced transformability and known genetic markers does not however exclude the possibility of some subtle, and normally undetected differences which may exist in the more transformable strains. These differences may become apparent only under the rigors of competence induction treatments. The nature of these could be either specific intramembraneous protein-LPS interactions not directly reflected in a particular genotype, or some cytoplasmic condition that may allow more efficient DNA establishment after its uptake. The possibility that DNA establishment is the primary source of more efficient plasmid transformation has been suggested by Hanahan (Hanahan, 1983), and is further supported by data presented in this report.

The relationship between transformational competence and various recombination pathways used by *E. coli* recipients is currently unclear. RecE recipients following plasmid DNA uptake have been found to be "hyperrecombinogenic" for ColE1-derived plasmids converting incoming plasmid monomers into segregationally unstable multimeric forms (Summers and Serratt, 1984; Clark and Cohen, 1986; Cohen and Silberstein, 1987). In this study, strain SC401 which utilizes the RecE pathway was found to be the most efficient in transformation. Recipients using the RecF pathway on the other hand, are unable to effectively recombine these plasmids maintaining them in their cytoplasm in monomeric form (Fishel et al., 1981). The Rec⁺ strains show intermediate plasmid recombinational efficiency, along with intermediate transformational efficiencies. These observations while interesting, do not constitute any proof that recombinational pathways affect plasmid transformational efficiency.

A comparison between the three different protocols of competence induction have shown that every *E. coli* strain tested, yielded significantly higher plasmid transformation efficiencies under the "RbCl-organic solvent" protocol than the "classic cold CaCl₂" method. Since all strains used responded similarly, the procedure must affect cellular components that are common to all strains. Time-course studies by Reusch et al., have shown that the "RbCl-organic solvent" protocol resulted in higher numbers of competent cells because they were produced at a faster rate than the Ca²⁺ protocol (Reusch

et al . , 1986) . No such transformational enhancement however , has been seen in the case of chromosomal DNA-mediated transformation . This is perhaps due to the limited ability of recipient cells , under any current protocol either to take up and/or to process into their genomes large , biologically active molecules of extracellular DNA .

The possibility that the organo-metal compound hexamine cobalt (HCo^{3+}) together with DMSO , and RbCl may facilitate the association of polyanionic DNA molecules with the polyanionic outer cell membrane has been previously raised by Hanahan (Hanahan , 1983 ; Hanahan 1987) . These compounds in the cold may affect either the conformation , or the accessibility of DNA uptake sites in the cell membrane to a greater extent than Ca^{2+} alone . This functional explanation of the increased transformational efficiency by the protocol of Hanahan , stems from the fact that neither of the above mentioned protocols of competence induction causes any gross changes in membrane lipid composition (Reusch et al . , 1986) .

The relatively low transformational efficiencies obtained by the protocol of Bergmans et al . , which involves the rapid pulsing of the preincubated cell-DNA mixture with high levels of Ca^{2+} and Mg^{2+} before the heat shock (Bergmans et al . , 1981) , suggest that the effect of Ca^{2+} on the bacterial envelope requires time to develop. The condition of time-dependent induction of competence by Ca^{2+} has been studied by Dagert and Ehrlich , who reported that the prolonged exposure of recipient cells to cold CaCl_2 often

results in more efficient plasmid transformations , than the standard protocol (Dagert and Ehrlich , 1979) . The rather short time of exposure (10 min) of the recipients to Ca^{2+} in the cold right before the heat shock , in the protocol of Bergmans et al , is apparently sufficient to render a number of cells competent for transformation .

Whether the sites for plasmid DNA uptake are also the sites for the uptake of other types of DNA is not clear . However , the effects of both non-transfecting chromosomal and competing phage DNAs on phage transfection suggest that while non transforming DNA reduces phage DNA uptake by virtue of its "bulk" , cotransfecting DNA competes very effectively for the presumptively limited number of DNA uptake sites present on the surfaces of recipient cells . A similar response of competing DNA on plasmid DNA transformation has been previously reported by Hanahan , (Hanahan , 1983) . It has been found by Hanahan that in the presence of HACo^{3+} , the addition of B_{12} to competent cells inhibited plasmid transformation and this suggested that DNA may be taken up by the B_{12} transport apparatus (Hanahan , 1983) . In this study an attempt was made to replicate these competition experiments in order to look further into the process of plasmid DNA uptake through the *btu* B transport sites . Cobalamin under all the conditions tested here was unable to effectively compete with either plasmid transformation or phage DNA transfection . Since the levels of the *btu* B protein are reportedly regulated

by the concentrations of exogenous cobalamin , which at high levels repress the expression of this protein (Kadner , 1978 ; Glass et al . , 1986) , one would expect that recipients grown in the presence of high levels of B₁₂ would be less transformable than their untreated counterparts , because of the reduction in the available DNA uptake sites on the recipients . No such reduction in transformability was seen when the recipients were grown in media supplemented with high levels of cobalamin , suggesting that the *Δu* B protein is not directly involved in DNA uptake .

Even though the nature of the components involved in external DNA uptake are presently undefined , the requirement of intact outer membrane proteins for the uptake of DNA was shown here by the inhibition of transformation following proteolytic treatment of competent cells . The experiments presented here show that the addition of trypsin to *E. coli* recipients prior to competence induction , drastically reduced transformation with plasmid DNA . Similar observations of reduced binding of DNA to isolated trypsin-treated outer membranes have been reported by Weston (Weston et al . , 1981) . The target of trypsin on the OM of recipients is unknown . It is possible that trypsin may act on some intramembranous aggregates of specific proteins and LPS located at cellular zones of adhesion which perhaps function as water filled channels to take up DNA into the cytoplasm of recipient cells (vanAlphen et al . , 1978 ; Hanahan , 1983) . Such particles

seem to appear on the surface of *E. coli* following cold CaCl_2 treatment (vanAlphen et al. , 1978) . It is unclear whether CaCl_2 treatment promotes these sites of DNA uptake , or it simply allows the proper exposure of DNA-binding proteins through the normally overlaying LPS layer . This question was addressed here by experiments involving the chemical removal of some of the sugar moieties of the LPS .

In the LPS's of *E. coli* and *S. typhimurium*, both the ten sugar "cores" and the repeating tetrasaccharides of the "O-side chains" are composed of sugars that are labile to periodate cleavage (Figure 11) . At relatively low concentrations of periodate the non essential "O side chains " are probably oxidized and stripped off , while at increasing concentrations of periodate the 2-Keto 3-deoxyoctonate (KDO) components of the core oligosaccharide , which are indispensable for cell viability may also be attacked . This perhaps is the cause of the rapid loss of cell viability observed at periodate concentrations above 10^{-3}M .

Periodate treatment of competent cells at moderate levels does not significantly alter transformation and transfection efficiencies of *E. coli* . This is in agreement with previously reported observations in which "rough" mutants of LPS of *E. coli* and *S. typhimurium* which lack the O-side chains of the LPS , were as transformable as their wild type "smooth" counterparts (Lederberg and Cohen , 1974) . In contrast , both *B. subtilis* and *H.*

influenzae transformations are severely reduced by periodate treatment (Polsinelli and Barlati, 1967; Ranhand and Lichstein, 1966), suggesting that the competent state in these organisms involves some periodate labile surface component. This component, at least in *H. influenzae* is presumably derived from the metabolism of inosine and lactate, and requires for full DNA binding activity active protein synthesis (Ranhand, 1969; Ranhand and Lichstein, 1969).

In *E. coli* competence is manifested very differently from other bacteria. Since there is no apparent correlation between the length of the polysaccharide side chain of the LPS and transformability in *E. coli* (MacLachlan and Sanderson, 1985), it can be argued that competence in this organism does not simply involve the Ca^{++} mediated "uncovering" of some DNA specific OM receptors. However, the rapid loss of transformability of *E. coli* due to the partial removal of LPS by EDTA (Taketo and Kuno, 1974) prior to the induction of competence and the restoration of transformation ability following the production of wild type LPS in some deep rough mutants (*rfa* D) (MacLachlan and Sanderson, 1985), suggest that LPS-outer membrane protein interaction could be a very important factor in transformation. Chemical treatments, or cellular mutations that would alter these interactions could affect the exposure of DNA binding sites, or could interfere with the uptake of DNA into recipient cells, without markedly reducing cell viability. The observed persistence of the transforming and

transfecting abilities of periodate-treated recipients may be due to the fact that the LPS-outer membrane protein interactions required for DNA uptake may not be sufficiently affected by such treatment.

The mechanism of penetration of DNA into recipient *E. coli* cells is currently unknown. Although it seems unlikely that a molecule as large as DNA can be taken up into a cell by passive diffusion, there is no real evidence that DNA is actively transported into cells. Kinetic studies by Weston et al. and this report show that external DNA becomes resistant to DNase only after a brief heat shock (Weston et al., 1981). During this process, a limited number of bound DNA molecules become internalized into either the periplasm or the cytoplasm.

A hypothetical account for the requirement of the heat pulse for the uptake of DNA into *E. coli*, suggests that rapid thermotropic phase shifts induced in the asymmetrical outer, and/or the more homogeneous cytoplasmic membrane lipid bilayers may allow DNA uptake (Sabelnikov and Domaradsky, 1979; van Die et al., 1983). Experimental data presented here confirms the idea that in the absence of heat shock the transforming DNA is bound to recipients but it is still exposed to the action of DNases. The transforming DNA becomes internalized and DNase resistant only after the heat pulse. DNA molecules may enter into the cytoplasm at envelope regions where they are aggregated by the cold membrane proteins meet the LPS layer during the very early stages of the heat shock (Verkleij, 1975; van Die et al., 1983). These hypothetical

"ports of entry" of DNA probably decay quickly , as the fluidity of the membrane returns to its more or less homogeneous state in the later stages of the heat shock (van-Die et al . , 1983) . Whether this uptake mechanism causes the permeabilization of the cytoplasmic membrane as well , is currently unknown . Although it appears that the plasma membrane is more resistant to CaCl_2 -mediated permeabilization (Brass et al . , 1983) . On the other hand , the effective induction of competence by *E. coli* spheroplast , at 0°C in the absence of Ca^{2+} (Henner et al . , 1973) , and the very low (3-fold less than normal) transformation efficiency of *E. coli* induced by Ca^{2+} at room temperature in the presence of the proton conductor carbonyl-m-chlorophenyl hydrazone (CCCP) (Taketo and Kuno , 1974 ; Sabelnikov and Domaradsky , 1979) , do not support the above hypothesis .

The extreme sensitivity of transformation to trypsin treatment suggests that the integrity of OM proteins may be important for DNA uptake (Weston et al . , 1981 ; MacLachan and Sanderson , 1985) . To assess the importance of overall protein synthesis in transformation and transfection , experiments were performed in which cellular protein synthesis was inhibited by antibiotics , and subsequently was reinitiated . The results of these experiments (Table 17) suggest that no new protein synthesis seems to be required in the recipient for efficient transformation and transfection to take place . This is in agreement with the recent data of Reusch et al . (Reusch et al . , 1986) .

In particular the absence of any significant reduction in transformation and

transfection efficiencies right after the inhibition of cellular protein synthesis by Cm , and to a lesser degree by Tet treatment suggests that recipients at this stage probably contain all the required components for both the binding and the establishment of the incoming DNA. A substantial enhancement in both transformation and transfection efficiencies was observed in several genetically different strains of *E. coli* , that recovered in LB broth for about 30 min after the action of the above bacteriostatic antimicrobial agents . The nature of these increases was transient , suggesting that both plasmid transformation and phage DNA-mediated transfection might be internally regulated , and are sensitive to unbalanced growth of the recipient . Radioactive labeling studies using labeled pBR322 DNA have shown that cells in recovery do not bind or take up more DNA than their untreated controls . Thus further supporting the idea that transformational efficiency is internally regulated .

Both Cm and to a lesser degree Tet stop protein synthesis without irreversibly damaging the cells (Lorian , 1971) . The former binds reversibly to the L16 protein of the bacterial ribosome and inactivates the peptidyl-transferase (Nierhaus and Nierhaus , 1973) , while the latter prevents the binding of aminoacyl-tRNA to the ribosomal A site (Gale et al , 1981) . About 60% of the bacterial protein synthesis stops in 1 min after the exposure of cells to 100 mg/ml Cm and 87 % after 10 min (Abdel-Sayed , 1987) . Also recipient cells appear to lack ribosomes in their cytoplasm after such

treatment (Lorian, 1985).

Even though the ColE1-derived plasmids, such as pBR322 require more or less a different set of host proteins for their replication than λ phage (Kingsbury and Helinski, 1973; Georgopoulos and Herskowitz, 1971; Sunshine et al., 1977; Saito and Uchida, 1978; Wickner, 1978; Friedman et al., 1984), the fact that the exposure of cells recovering from protein synthesis inhibition to both plasmid and phage DNAs, resulted in increased efficiencies of transformation and transfection, is suggestive of a mechanism which may be general in nature. The cell division inhibition by Dzu treatment did not affect plasmid transformation and phage DNA transfection. These results do not support the recent speculations of Saunders et al., (Saunders et al., 1987) that enhanced transformability might occur during cell division.

The increased efficiencies of transformation and transfection during recovery of recipients from protein synthesis inhibition cannot be explained. Protein synthesis has been implicated in cellular competence by Hanahan, because he believes that presence of transiently produced proteins may be involved with the compacting of DNA inside the cell, thus allowing for proper uptake of exogenous DNA. The intracellular levels of these proteins are perhaps related to the cell cycle (Hanahan, 1987). The observation that in any population of cells induced for competence, the same small group of cells is able to repeatedly transform (Sabelnikov et al., 1984), in addition to the fact that cells in stationary phase cannot be transformed, support this idea.

The enhancement of both transformation and transfection during recovery of the recipients from protein synthesis , suggests that DNA establishment , and not its uptake is the determining factor in transformation efficiency . Whether the observed increases in transformation and transfection efficiencies of these cells are due to the unbalanced production of some DNA condensing protein is unknown . Although the observed increases in the efficiency of transfection of large linear phages , like T7 , in both ExoV⁺ and ExoV⁻ strains of *E. coli* during recovery , are indicative of a more efficient establishment of this DNA in these cells . If DNA establishment involves some host protein-mediated condensing of incoming DNAs , then larger DNA molecules , requiring perhaps more such "processing" , would be more dependent on the presence of these proteins than smaller DNAs . More over , DNA molecules that may be already "processed" by virtue of their superhelicity would be less dependent of the presence of these proteins and thus transform a larger population of recipient cells . The more than 100-fold increase in transformation by small superhelical plasmids over other larger plasmids and phage DNAs , perhaps indicates that these plasmids are able to be established in recipients in which larger plasmids cannot . Intracellular conditions that affect the level of these proteins , most likely will also affect transformational ability . If the hypothetical mechanism of DNA establishment is correct , then the recipient cells that are in the "proper" stage of their cell cycle and contain these DNA condensing proteins that could mediate the

establishment of the incoming DNAs, will be transformed, while recipients that are not in this phase will not. The inhibition of protein synthesis would affect these competent cells minimally, since the condensing proteins were synthesized while they entered into the given cell cycle. During recovery from Cm treatment, perhaps either because of the synchronizing effect of the inhibition of protein synthesis, or because of temporary unbalanced cellular growth, more cells may produce these proteins, and thus are able to become transformed. The observed temporary nature of the increases in transformation and transfection of recovering cells might be due to the cellular "desynchronization" or because of the re-establishment of balanced growth in the recipient cells. Since the requirement of proper condensing of DNA may be minimal for the small mostly compacted plasmid DNAs like pBR322, and much more for larger and mostly loosely coiled DNAs, such as λ . Any effect that might result in the increasing in the number of cells that contain effective concentrations of DNA condensing proteins, will be much more evident with λ transfection, than with pBR322 transformation.

Given this speculation, mutations causing the reduction of this protein activity in the cell of would reduce phage DNA transfection much more than small plasmid transformation. The successful isolation of just such mutants that show efficient transformational ability for plasmid, but not with λ DNAs has been previously reported by Molchanova et al. (Molchanova et al., 1983). It would be interesting to see whether these mutants show the above

described recovery effect .

The transformation of recipients with large DNA fragments or plasmids must involve some form of DNA coiling . Whether this DNA coiling occurs during , or after its uptake is unknown , even though the limited dimensions of the DNA uptake sites favors the latter possibility. Further more this hypothesis may provide an insight to the nature of strain-dependent variations in transformability .

Inhibition and recovery from the action of the other antibacterial agents used in this study did not result in an increase of transfection and transformation efficiencies seen with Cm and Tet . Recovery from the effects of either DNA replication inhibition along with septation prevention and filamentation by the presence of Nal (Little and Mount , 1982) , or the inhibition of septation alone by Dzu , did not affect the efficiencies of transformation and transfection in *E. coli* . Since nalidixic acid is a good inducer of the SOS system of *E. coli* , and its addition had no detectable effect , it is probable that the SOS system of repair does not play a significant role in transformation and transfection in this organism .

E. coli and other halotolerant bacteria are able to adapt to conditions of extreme osmolarity by different mechanisms (Gowrishankar , 1985) . In hypotonic media *E. coli* maintain osmotic balance across its cytoplasmic membrane by the synthesis of membrane-derived oligosaccharides (MDO) (Kennedy , 1982) . On the other hand , in hypertonic media , the cellular turgor

pressure is stabilized by the activation of a high affinity K^+ uptake protein (*kds* locus) , along with the cytoplasmic accumulation of organic osmoprotectants. These include proline and the trimethylated glycine derivative betaine (Perroud and Le Rudulier , 1985 ; Landfald and Strom , 1986) .

Mutant strains of *E. coli* with abnormal MDO production usually display normal transformational efficiencies (Dr Kennedy personal communication) . Similarly this study shows that osmoprotection of recipient cells does not affect in either way the efficiency of either transformation with plasmid DNA , or transfection with phage DNA . This suggests on the one hand , that the previously proposed requirement of limited plasmolysis of the recipient for efficient plasmid transformation (Bukau et al . , 1985) may not be necessary, and on the other , that cytoplasmic establishment is the determining factor in both of these processes .

LITERATURE CITED

1. Abdel-Sayed , S . 1987. Transport of chloramphenicol into sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa*. *J . of Antimicrobial Chemotherapy* **19** : 7-20 .
2. Akrigg , A . , and S . R . Ayad . 1970 . Studies on the competence - inducing factor of *Bacillus subtilis*. *Biochem. J.* **117** : 397-403 .
3. Albritton , W . L . , J . W . Bendler , J . K . Setlow . 1981 . Plasmid transformation in *Haemophilus influenzae*. *J. Bacteriol.* **145** : 1099-101.
4. Ames , B . N . , and D . T . Dubin . 1960 . The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid . *J. Biol. Chem.* **235** : 769-775 .
5. Amundsen , S . K . , A . F . Taylor , A . M . Chaudhury , and G . R . Smith . 1986 . *BacD* : The gene for an essential third subunit of exonuclease V . *Proc. Natl. Acad. Sci. USA* **83** : 5558-5562 .
6. Appleyard , R . K . 1954 . Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K-12 . *Genetics* **39** : 440-452 .
7. Barany , F . , M . E . Kahn , and H . O . Smith . 1983 . Directional transport and integration of donor DNA in *Haemophilus influenzae* transformation

- Proc. Natl. Acad. Sci. USA* 80:7274-7278.
8. Barbour, S. D., H. Nagaishi, A. Templin, and A. J. Clark. 1970. Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. II. Rec⁺ revertants caused by indirect suppression of Rec⁻ mutations. *Proc. Natl. Acad. Sci. USA* 67:128-135.
 9. Barbour, S. D., and A. J. Clark. 1970. Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. I. Enzymatic activity associated with recB⁺ and recC⁺ genes. *Proc. Natl. Acad. Sci. USA* 65:955-961.
 10. Barron, A., G. May, E. Bremer, and M. Villarejo. 1986. Regulation of envelope protein composition during adaptation to osmotic stress in *Escherichia coli*. *J. Bacteriol.* 167:433-438.
 11. Basset, C. L., and S. R. Kushner. 1984. Exonucleases I, II and V are required for stability of ColE1-related plasmids in *Escherichia coli*. *J. Bacteriol.* 157:661-664.
 12. Bayer, M. E. 1968a. Areas of adhesion between wall and membrane of *Escherichia coli*. *J. Gen. Microbiol.* 53:395-404.
 13. Bayer, M. E. 1968b. The adsorption of bacteriophages to adhesions between wall and membrane of *Escherichia coli*. *J. Virol.* 2:346-356.
 14. Bayer, M. E., M. Dolack, and E. Houser. 1977. Effects of lipid phase transition on the freeze-leaved envelope of *Escherichia coli*. *J. Bacteriol.* 129:1563-1573.

15. Benzinger, R., I. Kleber, and R. Huskey. 1971. Transfection of *Escherichia coli* spheroplasts. I. General facilitation of double-stranded deoxyribonucleic acid infectivity by protamine sulfate. *J. Virol.* **7**: 646-650.
16. Benzinger, R., L. W. Enquist, and A. Skalka. 1975. Transfection of *Escherichia coli* spheroplasts. V. Activity of the *recBC* nuclease in *rec*⁺ and *rec*⁻ spheroplasts measured with different forms of bacteriophage DNA. *J. Virol.* **15**: 861-871.
17. Benzinger, R. 1977. Modern Trends in Bacterial Transformation and Transfection Portoles, A., R. Lopez, and M. Espinosa (eds.), pp. 87-92. Amsterdam, North-Holland.
18. Benzinger, R. 1978. Transfection in Enterobacteriaceae and its application. *Microbiol. Rev.* **42**: 194-236.
19. Berg, D. E., A. Jackson, and E. Mertz. 1974. Isolation of a λ dv plasmid carrying the bacterial *gal* operon. *J. Virol.* **14**: 1063-1069.
20. Bergan, T., and I. B. Carlsen. 1985. Effect of antibiotics eliminated by first order kinetics. *J. Antimicrobial Chemotherapy* **15**: Supp. A, 147-152.
21. Bergmans, H. E. N., I. M. Van Die, and W. P. M. Heeoksrt. 1981. Transformation in *Escherichia coli*: Stages in the process. *J. Bacteriol.* **146**: 564-570.
22. Bergmans, H. E. N., D. H. Kooijman, and W. P. M. Heeoksrt.

- 1980 . Cotransformation of linear chromosomal DNA and plasmid DNA in *Escherichia coli* . *FEMS Mikrobiol. Lett.* **9** : 211-214 .
23. Biek , D . P . , and S . N . Cohen . 1986 . Identification and characterization of *recD* a gene affecting plasmid maintenance and recombination in *Escherichia coli* . *J. Bacteriol.* **167** : 594-603 .
- 24 . Birnboim , H . C . , and J . Doly . 1979 . A rapid alkaline extraction procedure for screening recombinant plasmids . *Nucleic Acids Res.* **7** : 1513- 1525 .
25. Biswas , G . D . , T . Sox , and P . F . Sparling . 1977 . Factors affecting genetic transformation of *Neisseria gonorrhoea* . *J. Bacteriol.* **129** : 983-992 .
26. Biswas , G . D . , J . Graves , R . Schwalbe , and P . F . Sparling . 1986 . Construction of isogenic Gonococcal strains varying in the presence of a 4 . 2- Kilobase cryptic plasmid . *J. Bacteriol.* **167** : 685-694 .
- 27 . Bolivar , F . , and K . Backman . 1979 . Plasmids of *Escherichia coli* as cloning vectors . *Methods Enzymol.* **68** : 245-267 .
28. Brass , J . M . , U . Ehmann , and B . Bukau . 1983 . Reconstitution of maltose transport in *Escherichia coli* : Conditions affecting import of maltose-binding protein into the periplasm of calcium treated cells . *J. Bacteriol.* **155** : 97-106 .
- 29... Brass , J . M . , B . Bukau , and M . D . Manson . 1984 . Calcium-induced permeabilization of the outer membrane of *Escherichia coli* : A method

- for introducing biologically active macromolecules into the periplasmic space . *Mikrobiology* **17A** : 66-70 .
30. Brass , J . M . , C . F . Higgins , M . Foley , P . A . Rugman , J . Birmingham , and P . B . Garland . 1986 . Lateral diffusion of proteins in the periplasm of *Escherichia coli* . *J. Bacteriol.* **165** : 787-794 .
 31. Brown , M . G . M . , A . Weston , J . Saunders , and G . O . Humphreys . 1979 . Transformation of *Escherichia coli* C600 by plasmid DNA at different phases of growth . *FEMS . Mikrobiol. Lett.* **5** : 219-222 .
 32. Bukau , B . , J . M . Brass , and W . Boos . 1985 . Ca^{2+} - induced permeabilization of the *Escherichia coli* outer membrane : Comparison of transformation and reconstitution of binding-protein-dependent transport . *J. Bacteriol.* **163** : 61-68
 33. Bursztyn , H . , V . Sgamarella , O . Clifferri , and J . Lederberg . 1975 . Transfectability of rough strains of *Salmonella typhimurium* . *J. Bacteriol* **124** : 1630-1634 .
 34. Cairney , J . , C . F . Higgins , I . R . Booth . 1985 . Proline uptake through the major transport system of *Salmonella typhimurium* is coupled to sodium ions . *J. Bacteriol.* **160** : 22-27 .
 35. Cahn , F . H . , and M . S . Fox . 1968 . Fractionation of transformable bacteria from competent cultures of *Bacillus subtilis* on Renograffin gradients . *J. Bacteriol.* **95** : 867-875 .
 36. Canosi , U . , G . Morelli , and T . A . Trautner . 1978 . The relationship
-

- between molecular structure and transformation efficiency of some *S. aureus* plasmids isolated from *B. subtilis*. *Mol. Gen. Genet.* **166** : 259-267 .
37. Caster , J . H . , E . H . Postel , and S . H . Goodgal . 1970 . Competence mutants : The isolation of transformation in deficient strains of *Haemophilus influenzae*. *Nature* (London) **227** : 515-517 .
38. Ceglowski , P . , M . Kawczynski , and W . T . Dobrzanski . 1980 . Purification and properties of deoxyribonucleic acid-binding factor isolated from the surface of *Streptococcus sanguis* cells . *J. Bacteriol.* **141** : 1005-1014 .
39. Clark , A . J . 1973 . Recombination deficient mutants of *Escherichia coli* and other bacteria . *Ann. Rev. Genet.* **7** : 67-86 .
40. Clark , P . O . , and F . R . Leach . 1980 . Stimulation of *Bacillus subtilis* transformation by spermidine . *Mol. Gen. Genet.* **178** : 21-25 .
41. Clark , A . J . , A . Cohen . 1986 . Synthesis of linear plasmid multimers in *Escherichia coli* K-12 . *J. Bacteriol.* **167** : 327-335 .
42. Claverys , J . P . , M . Roger , and A . M . Sicard . 1980 . Excision repair of mismatched base pairs in transformation of *Streptococcus pneumoniae*. *Mol. Gen. Genet.* **178** : 191-201 .
43. Cohen , S . N . , A . C . Y . Chang , and L . Hsu . 1972 . Nonchromosomal antibiotic resistance in bacteria : Genetic transformation by R-factor DNA . *Proc Natl. Acad. Sci. U.S.A.* **69** : 2110-2214 .

44. Cohen , A . , Z . Silberstein . 1987 . Synthesis of linear multimers of oriC and pBR322 derivatives in *Escherichia coli* K-12 : Role of recombination and replication functions . *J. Bacteriol.* **169** : 3131-3137 .
45. Coleman , G . 1967 . Studies on the regulation of extracellular enzyme formation by *Bacillus subtilis*. *J. Gen. Microbiol.* **49** : 421-431 .
46. Contente , S . , and D . Dubnau .1979 . Characterization of plasmid transformation in *Bacillus subtilis* : Kinetic properties and the effect of DNA conformation . *Mol. Gen. Genet.* **167** : 251-258 .
47. Cosloy , S . D . , and M . Oishi . 1973a . Genetic transformation in *Escherichia coli* K-12 . *Proc. Natl. Acad. Sci. USA* **70** : 84-87.
48. Cosloy , S . D . , and M . Oishi . 1973 b . The nature of the transformation process in *Escherichia coli* . *Mol. Gen. Genet.* **124** : 1-10 .
49. Colton, F . A . , G . Wilkinson .1972 . Advanced Inorganic Chemistry, 3rd ed . N.Y . Interscience pp. 189 and 206 . John Wiley and Sons N . Y .
50. Cullis , P.R . , and B . de Kruijff . 1979 . Lipid polymorphism and the functional roles of lipids in biological membranes . *Biochim. Biophys . Acta* **559** : 399-420 .
51. Dagert , M . , and S.D . Ehrlich , 1979 . Prolonged incubation in calcium -chloride improves the competence of *Escherichia coli* cells . *Gene* **6** : 23-28 .
- 52 . Danner , D . B . , R . A . Deich , K . L . Sisco , and H . O . Smith .1980 . An 11-base pair sequence determines the specificity of DNA uptake in

- Haemophilus* transformation . *Gene* **11** : 311-318 .
53. Davis , R . W . , D . Bolstein , J . R . Roth . 1980 . Advanced Bacteriological Genetics . pp. 116-125 Cold Spring Harbor . Cold Spring Harbor . N.Y
 54. Deich , R . A . , H . O . Smith . and D . B . Danner . 1981 . Genetic Transformation . *Ann. Rev. Biochem.* **50** : 41-68 .
 55. de Lencastre , H . , and P . J . Piggot . 1979 . Identification of different sites of expression for *spo* loci by transformation of *Bacillus subtilis* . *J. Gen. Microbiol.* **114** : 377-389 , 1.
 56. Di Masi , D . R . , J . C . White , C . A . Schnaitman , and C . Bradbeer . 1973 . Transport of vitamin B12 in *Escherichia coli* : Common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope . *J. Bacteriol.* **115** : 506-513 .
 57. Dougherty , J . J . , A . Asmus , A . Tomasz . 1979 . Specificity of DNA uptake in genetic transformation of Gonococci . *Biochem. Biophys. Res Commun.* **86** : 97- 104 .
 58. Dubnau , D . , and C . Cirigliano . 1972 . Fate of transforming DNA following uptake by competent *Bacillus subtilis* : IV . The endwise attachment and uptake of transforming DNA . *J. Mol. Biol.* **64** : 31-46
 59. Dubnau , D . , and C . Cirigliano . 1973 . Fate of transforming DNA following uptake by competent *Bacillus subtilis* : VI . Non-covalent association of donor and recipient DNA . *Mol. Gen. Genet.* **120** : 101-106 .

60. Dubnau , D . , and C . Cirigliano . 1974 . Genetic characterization of recombination-deficient mutants of *Bacillus subtilis* . *J. Bacteriol.* **117**: 488- 493 .
61. Dubnau , D . , 1976 . Genetic Transformation of *Bacillus subtilis* ; a review with emphasis on the recombination mechanisms . *Mikrobiology* - 1976. pp. 14-27 , Am . Soc . Microbiol . Washington DC .
62. Dubnau , D . , M . Albano , and J . Hahn . 1987 . Expression of competence genes in *Bacillus subtilis* . *J. Bacteriol.* **169** : 3110-2117.
63. Dunlap , Y . J . , and L . N . Csonka . 1985 . Osmotic regulation of L-proline transport in *Salmonella typhimurium* . *J. Bacteriol.* **163** : 296-304 .
64. Dykstra , C . C . , D . Prasher , and S . R . Kushner . 1984 . Physical and biochemical analysis of the cloned *recB* and *recC* genes of *Escherichia coli* K-12 . *J. Bacteriol.* **157** : 21-27 .
65. Eisenstark , A . 1977 . Genetic recombination in bacteria . *Ann. Rev. Genet.* **11** : 369- 396 .
66. Enea , V . , G . F . Vovis , and N . D . Zinder . 1975 . Genetic studies with heteroduplex DNA of bacteriophage f1 . Asymmetric segregation , base correction and implications for the mechanism of genetic recombination . *J. Mol. Biol.* **96** : 495 .
67. Ephrussi-Taylor, H . , and B . A . Freed . 1964 . Incorporation of thymidine and amino acids into deoxyribonucleic acid and acid-insoluble cell

- structures in pneumococcal cultures synchronized for competence to transform . *J . Bacteriol .* **87** : 1211-1215 .
- 68 .Erickson , J . R . 1970 . Current Topics in Microbiology and Immunology . **53** : 149 . Springer Verlag .
- 69 .Falkner, I . , and C . O . Wyss . 1964 . A substance produced by competent *Bacillus cereus* 569 cell that affects transformability . *Biochim . Biophys . Res . Commun .* **16** : 94-102 .
- 70 .Felsenfeld , G . , and S . Huang . 1960 . The interactions of polynucleotides with metal ions , amino acids and polyamines . *Biochim . Biophys . Acta* **37** : 425-433 .
- 71 .Ferris , F . G . , and T . J . Beveridge . 1986 . Physicochemical roles of soluble metal cations in the outer membrane of *Escherichia coli* K-12 . *Can . J . Microbiol .* **32** : 594-601 .
- 72 .Fishel , P . A . , A . James , and A . Kolodner . 1981 . *recA* -independent general genetic recombination of plasmids . *Nature* (London) **294** : 184-186 .
- 73 .Flock , J . I . 1978 . Transfection with replicating DNA from the temperate *Bacillus* bacteriophage ϕ 105 and with T4 -ligase treated ϕ 105 DNA : The importance in transfection of being longer than genome-length . *Mol . Gen. Genet .* **163** : 7-15 .
- 74 .Fox , M . S . , and R . D . Hotchkiss . 1957 . Initiation of bacterial transformation . *Nature* (London) **179** : 1322-1325 .

75. Fox , M . S . 1978 . Some features of genetic recombination in prokaryotes . *Ann Rev. Genet.* **12** : 47-68 .
- 76... Friedman , D . I . , E . R . Olson , C . Georgopoulos , K . Tilly , I . Herskowitz and F . Banuett . 1984 . Interactions of bacteriophage λ and host macromolecules in the growth of bacteriophage λ . *Microbial Reviews* **48** : 299-325 .
77. Georgopoulos , C . P . , and I . Herskowitz .1971. The Bacteriophage Lambda. A . D . Hersey (ed .) , pp. 553-564 Cold Spring Harbor Laboratory , Cold Spring Harbor N.Y .
78. Gawrishankar, J . 1985 . Identification of osmoreponsive genes in *Escherichia coli* : Evidence for participation of potassium and proline transport systems in osmoregulation . *J. Bacteriol.* **164** : 434- 445 .
79. Gillen , J . R . , D . K . Willis , and A . J . Clark . 1981 . Genetic analysis of the RecE pathway of genetic recombination in *Escherichia coli* K-12 . *J. Bacteriol.* **145** : 521-532
80. Glass , R . E . , A . J . P . Fletcher , A . P . Pugsley . 1986 . Investigation of the regulation of the *Escherichia coli* *btuB* gene using operon fusions . *J. Gen. Microbiol.* **132** : 2643-2646 .
81. Goldmark , P . J . , and S . Linn . 1972 . Purification and properties of the *recBC* DNase of *Escherichia coli* K-12 . *J. Biol. Chem.* **247** : 1849-1860 .
82. Goodgal , H . S . , and R . M . Herriott . 1961. Studies of transformation of

- Haemophilus influenzae* : competence . *J. Gen. Physiol.* **44** : 1201-1227.
83. Goodgal , H . S . 1982 . DNA uptake in *Haemophilus* transformation . *Ann. Rev. Genet.* **16** : 169-192 .
84. Goodgal , H . S . , and M . F . Concino . 1982 . *Haemophilus* polypeptides involved in deoxyribonucleic acid uptake detected by cellular surface protein iodination . *J. Bacteriol.* **148** : 220-231 .
85. Gosule , L . C . , and J . A . Schellman . 1976 . Compact form of DNA induced by spermidine . *Nature* (London) **259** : 333-335 .
86. Graham , B . J . , and C . A . Istock . 1978 . Genetic exchange in *Bacillus subtilis* in soil . *Mol. Gen. Genet.* **166** : 287-290 .
87. Green , D . M . 1966 . Physical and genetic characterization of sheared infective SP82 bacteriophage DNA . *J. Mol. Biol.* **22** : 15-22 .
88. Grist , R . W . , and L . O . Butler . 1981 . The use of M1 medium in transformation of *Streptococcus pneumoniae* . *J. Gen. Microbiol.* **127** : 147- 154 .
89. Grist , R . W . and L . O . Butler . 1983 . Effect of transforming DNA on growth and frequency of mutation of *Streptococcus pneumoniae* . *J. Bacteriol.* **153** : 153-162 .
90. Gromkova , R . , and S . H . Goodgal . 1981 . The uptake of plasmid deoxyribonucleic acid by *Haemophilus* . *J. Bacteriol.* **146** : 79-84
91. Hanahan , D . 1983 . Studies on transformation of *Escherichia coli*

- with plasmids . *J. Mol. Biol.* **166** : 552-580 .
92. Hanahan , D . 1985 . DNA Cloning Vol . I , A practical approach . D . M . Glover (ed.) , pp . 109-135 . IRL Press Oxford U . K .
 93. Hanahan , D . 1987 . *E . coli* and *S . typhimurium* , Cellular and Molecular Biology , F . C . Neidhardt , J . L . Ingraham , K . Brooks Low , B Magasanik , M . Schaechter , and H . E . Umbarger (eds) Vol . 2 , pp . 1177-1183 . American Society for Microbiology , Washington D . C .
 94. Hanawalt , P . C . , P . K . Cooper , A . K . Ganesan , and C . A . Smith . 1979 DNA repair in bacteria and mammalian cells . *Ann. Rev. Biochem.* **48** : 783-836 .
 95. Harlos , K . , and H . Eibl . 1980 . Influence of calcium on phosphatidylglycerol . Two separate lamellar structures . *Biochemistry.* **19** : 895- 899 .
 96. Henner , W . D . , I . Kleber . and R . Benzinger . 1973 . Transfection of *Escherichia coli* spheroplasts . III . Facilitation of transfection and stabilization of spheroplasts by different basic polymers . *J. Virol.* **12** : 741-47 .
 97. Hobot , J . A . , E . Carleman , W . Villiger , and E . Kellenberger . 1984 . Periplasmic gel : New concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods . *J. Bacteriol.* **160** : 143-152 .
 98. Horii , Z . I . , and A . J . Clark . 1973 . Genetic analysis of the RecF
-

- pathway of genetic recombination in *Escherichia coli* K-12 . *J. Mol. Ecol.* **80** : 327-344 .
99. Humphreys , G . O . , A . Weston , G . M . G . Brown , and J . R . Saunders .
1979 . Plasmid Transformation of *Escherichia coli* , S . W . Glover , and L
(eds.) , In Transformation 1978 . pp . 287-312 Cotswold Press . Oxford
 100. Ingraham , , J . L . , C . A . Carlson , L . S . Pierson , , and J . J . Rosen .
1983 . *Pseudomonas stutzeri* and related species undergo natural
transformation . *J. Bacteriol.* **153** : 93-99 .
 101. Joenje , H . , and G . Venema . 1975 . Different nuclease activities in
competent and noncompetent *Bacillus subtilis* . *J. Bacteriol.* **122** :
25-33 .
 102. Jones , I . M . , S . B . Primrose , A . Robinson , and D . C . Ellwood .
1981 . Effect of growth rate and nutrient limitation on the transformability
of *Escherichia coli* with plasmid deoxyribonucleic acid . *J. Bacteriol.*
146 : 841-846
 103. Juni , E . , and G . A . Heym . 1980 . Transformation assay for
identification of psychrotrophic achromobacters . *Appl. Environ.*
Mikrobiol. **40** : 1106-1114 .
 104. Kadner , R . J . 1978 . Repression of synthesis of the vitamin B12 in
Escherichia coli . *J. Bacteriol.* **136** : 1050-1057 .
 105. Kahn , M . E . , F . Barany , H . O . Smith . 1983 . Transformasomes :
Specialized membranous structures that protect DNA during *Haemophilus*

- transformation . *Proc. Natl. Acad. Sci. USA*. **80** : 6927-6931 .
- 106 Kahn , M . E . , G . Maul , and S . H . Goodgal . 1982 . Possible mechanism of donor DNA binding and transport in *Haemophilus* . *Proc. Natl. Acad. Sci. USA* **79** : 6370-6374 .
107. Kaiser , A . D . , H . Tabor , and C . W . Tabor . 1963 . Spermine protection of coliphage λ DNA against breakage by hydrodynamic shear . *J. Mol. Biol.* **6** : 141-147 .
108. Kawaji , H . , T . Mizuno , and S . Mizushima . 1979 . Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins O-8 and O-9 of *Escherichia coli* K-12 . *J. Bacteriol.* **140** : 843-47.
109. Kellenberger , E . , and J . Sechaud . 1957 . Electron microscopical studies of phage multiplication . II . Production of phage-related structures during multiplication of phages T2 and T4 . *Virology*. **3** : 256-274 .
110. Kennedy , E . P . 1982 . Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli* . *Proc. Natl. Acad. USA* . **79** : 1092- 1095
111. Kingsbury , D . T . , and D . R . Hellinski . 1973 . Temperature-sensitive mutants for the replication of plasmids in *Escherichia coli* : Requirement for deoxyribonucleic acid polymerase I . in the replication of plasmid ColE1 . *J. Bacteriol.* **114** : 1116-1124 .

112. Kushner, S. R., H. Nagaishi, A. Templin, and A. J. Clark. 1971. Genetic recombination in *Escherichia coli*: The role of exonuclease I. *Proc. Natl. Acad. Sci. USA*. **68** : 824-827.
113. Kushner, S. R., H. Nagaishi, and A. J. Clark. 1972. Indirect suppression of *recB* and *recC* mutations by exonuclease I deficiency. *Proc. Natl. Acad. Sci. USA*. **69** : 1366-1370.
114. Kushner, S. R. 1978. In Genetic Engineering. Boyer, H. S. B and S. Nicosia (eds.) pp. 17-23. Elsevier, Amsterdam, Holland.
115. Labischinski, H., G. Barnickel, H. Bradaczek, D. Naumann, E. T. Rietschel and P. Giesbrecht. 1985. High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. *J. Bacteriol.* **162** : 9-20.
116. Lacks, S., B. Greenberg, and M. Neuberger. 1975. Identification of a deoxyribonuclease implicated in genetic transformation of *Diplococcus pneumoniae*. *J. Bacteriol.* **123** : 222-232.
117. Lacks, S. A., 1977. Binding and Entry of DNA in Bacterial Transformation. Reissig, J. L. (ed) *Microbial Interactions Series E*. Vol. 3 pp. 177-232. Chapman and Hall, London.
118. Lacks, S. 1979. Uptake of circular deoxyribonucleic acid and mechanism of deoxyribonucleic acid transport in genetic transformation of *Streptococcus pneumoniae*. *J. Bacteriol.* **138** : 404-409.

119. Laimins , L . A . , D . B . Rhoads , and W . Epstein . 1981 . Osmotic control of *kds* operon expression in *Escherichia coli* . *Proc. Natl. Acad. Sci. USA* . **78** : 464-468 .
120. Landfald , B . , and A . R . Strom . 1986 . Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli* . *J. Bacteriol* . **165** : 849-855 .
121. Levine , J . S . , and N . Strauss . 1965 . Lag period characterizing the entry of transforming DNA into *Bacillus subtilis* . *J. Bacteriol* . **89** : 281-287 .
122. LeClerc , J . E . , and J . K . Setlow . 1975 . Single-strand regions in the deoxyribonucleic acid of competent *Haemophilus influenzae* . *J. Bacteriol* . **122** : 1091-1102 .
123. Lederberg , E . M . , and S . N . Cohen . 1974 . Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid . *J. Bacteriol* **119** : 1072-1074 .
124. Ledoux , L . , P . Roleants , Y . Konvalinkova , M . Mergeay , and P . F . Lurquin . 1976 . DNA uptake by *Streptomyces* species . *Biochim. Biophys. Acta* . **442** : 117-122 .
125. Leonard , C . G . , and R . M . Cole . 1972 . Purification and properties of Streptococcal competence factor isolated from chemically defined medium . *J. Bacteriol* . **110** : 273-280 .
126. Le Rudulier , D . , and L . Bouillard . 1983 . Glycine betaine , an osmotic

- effector in *Klebsiella pneumoniae* and other members of the Enterobacteriaceae . *Appl. Environ. Microbiol.* **46** : 152-159 .
127. Le Rudulier , D . , A . R . Strom , A . M . Dandekar , L . T . Smith , and R . C . Valentine . 1984 . Molecular biology of osmoregulation . *Science* **224** : 1064-1068 .
128. Letellier , L . , and B . Labedan . 1984 . Involvement of envelope-bound calcium in the transient depolarization of the *Escherichia coli* cytoplasmic membrane induced by bacteriophage T4 and T5 adsorption . *J. Bacteriol.* **157** : 789-794 .
129. Lindberg , M . , J . E . Sjostrom , and T . Johanson . 1972 . Transformation of chromosomal and plasmid characters in *Staphylococcus aureus* . *J. Bacteriol.* **109** : 844-847 .
130. Little , J . W . , and D . W . Mount . 1982 . The SOS regulatory system of *Escherichia coli* . *Cell* **29** : 11-22 .
131. Lloyd , R . G . , and C . Buckman . 1985 . Identification and genetic analysis of *sbc* mutations in commonly used *recBC sbcB* strains of *Escherichia coli* K-12 . *J. Bacteriol.* **164** : 836-844 .
132. Lloyd , R . G . , and A . Thomas . 1983 . On the nature of the RecBC and RecF pathways of conjugal recombination in *Escherichia coli* . *Mol. Gen. Genet.* **190** : 156-161 .
133. Lloyd , R . G . , F . E . Benson , and C . E . Shurvinton . 1984 . Effect of *run* mutations on recombination and repair in *Escherichia coli* K-12 .

- Mol. Gen. Genet.* **194** : 303-309 .
134. Lopez , R . , A . Tapis , and A . Portoles . 1975 . The influence of glutamic acid and arginine on the competence of *Bacillus subtilis* growing in a chemostat . *Mol. Gen. Genet.* **136** : 87-94 .
 135. Lorian , Y . 1971 . The mode of action of antibiotics on gram-negative bacilli . *Arch. Intern. Med.* **128** : 623-632 .
 136. Lorian , Y . 1985 . Low concentration of antibiotics . *J. Antimicrobial Chemotherapy* **15** Suppl. A : 15-26 .
 137. Low , K . B . , and D . D . Porter . 1978 . Modes of gene transfer and recombination in bacteria . *Ann. Rev. Genet.* **12** : 249-287 .
 138. Lugtenberg , B . , R . Peters , H . Bernheimer , and W . Berendesen 1976. influence of culture conditions and mutations on the composition of the outer membrane proteins of *Escherichia coli*. *Mol. Gen. Genet* **147** : 251-262 .
 139. Lugtemerg , B . , and L . van Alphen . 1983 . Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria . *Biochim. Biophys. Acta.* **737** : 51-115 .
 140. MacLachlan , P . R . , and K . E . Sanderson . 1985 . Transformation of *Salmonella typhimurium* with plasmid DNA : Differences between rough and smooth strains . *J. Bacteriol.* **161** : 442-445 .
 141. Mandel , M . , and A . Higa . 1970 . Calcium-dependent bacteriophage DNA infection . *J. Mol. Biol.* **53** : 159-162 .
-

142. Maniatis , T. , E . F . Fritsch , and J . Sambrook . 1982 . Molecular cloning a laboratory manual . Cold Spring Harbor Laboratory . Cold Spring Harbor, New York .
143. Markal , R . P. , B . Werninghaus , and E . A . Evans Jr . 1964 . The formation of λ bacteriophage by λ DNA in disrupted cell preparations . *Proc. Natl. Acad. Sci. USA* . **51** : 1172-1178 .
144. Marmur , J . 1961 . A procedure for the isolation of deoxyribonucleic acid from microorganisms . *J. Mol. Biol.* . **3** : 208-218 .
- 145.. Mazza , G . , A . Fortunato , E . Ferrari , U . Canesi , A Falaschi , and M . Polsinelli . 1975 . Genetic and enzymatic studies on the recombination process in *Bacillus subtilis* . *Mol. Gen. Genet.* . **136** : 9-30 .
146. Molchanova , E . S . , N . A . Likhacheva , A . A . Kim , and B . N . Ilyashenko 1983 . The study of *Escherichia coli* K-12 mutants with the enhanced efficiency of plasmid transformation . *Genetika* . **19** : 375-320 .
147. Morrison , D . A . , and W . R . Guild . 1972 . Activity of deoxyribonucleic acid ragments of defined size in *Bacillus subtilis* transformation . *J. Bacteriol.* . **112** : 220-223 .
148. Morrison , D . A . , and W . R . Guild . 1973 . Transformation and deoxyribonucleic acid size : Extent of degradation on entry varies with size . *J. Bacteriol.* . **112** : 1157-1168 .
149. Morrison , D . A . , and M . F . Baker . 1979 . Competence for genetic

- transformation in pneumococcus depends on synthesis of a small set of proteins . *Nature* (London) **282** : 215-217 .
150. Morrison , D . A . , B . Mannarelli , and M . N . Vijayakumar . 1982 .
Competence for transformation in *Streptococcus pneumoniae*: inducible high-capacity system for genetic exchange . Microbiology 1982 . D . Schlessinger (ed.) , American Society for Microbiology , Washington DC
151. Muhlradt , P . F . , J . Menzel , J . R . Golecki , and V . Speth . 1973 .
Outer membrane of *Salmonella*, sites of export of newly synthesized lipopolysaccharide on the bacterial surface . *Eur. J. Biochem.* **35** : 471-481 .
152. Nakayama , H . , K . Nakayama , N . Isono , Y . Nakayama , and P . C . Hanawalt . 1984 . Isolation and genetic characterization of a thymineless death-resistant mutant of *Escherichia coli* K-12 : Identification of a new mutation (*recQ1*) that blocks the RecF recombination pathway . *Mol. Gen. Genet.* **195** : 474-480 .
153. Nester, E . W . 1964 . Penicillin resistance of competent cells in deoxyribonucleic acid transformation of *Bacillus subtilis* . *J. Bacteriol.* **87** : 867-875 .
154. Nierhaus , D . , and K . H . Nierhaus . 1973 . Identification of the chloramphenicol-binding protein in *Escherichia coli* ribosomes by partial reconstitution . *Proc. Natl. Acad. Sci. USA* **70** : 2224-2228 .

155. Nikaido , H . , and M . Vaara . 1985 . Molecular basis of bacterial outer membrane permeability . *Microbiological Reviews* **49** : 1-32 .
156. Nikaido , H . , and T . Nakae . 1979 . The outer membrane of gram-negative bacteria . *Adv. Microb. Physiol.* **20** : 163- 250 .
157. Norgard , M . Y . , K . Keem , and J . J . Monahan . 1978 . Factors affecting the transformation of *Escherichia coli* strain X1776 by pBR322 plasmid DNA . *Gene* **3** : 279-292 .
158. Norgard , M . Y . , and T . Imaeda . 1978 . Physiological factors involved in transformation of *Mycobacterium smegmatis* . *J. Bacteriol.* **133** : 1254-1262 .
159. Noteborn , M . , G . Venema , and J . Kooistra . 1981 . Effect of ethelenediaminetetraacetic acid on deoxyribonucleic acid entry and recombination in transformation of a wild-type strain and a *rec*-1 mutant of *Haemophilus influenzae* . *J. Bacteriol.* **145** : 1189-1195 .
160. Oishi , M . 1969 . An ATP-dependent deoxyribonuclease from *Escherichia coli* with a possible role in genetic recombination . *Proc. Natl. Acad. Sci. USA* **64** : 1291-1299 .
161. Oishi , M . , and R . M . Irbe . 1977 . Modern Trends in Bacterial Transformation and Transfection . Portoles , A . , R . Lopez , M . Espinosa (eds.) pp. 341-348 Elsevier , Amsterdam North-Holland .
162. Oishi , M . , and S . D . Cosloy . 1972 . The genetic and biochemical basis of the transformability of *Escherichia coli* K-12 . *Biochem. Biophys*

- Res. Comm.* **49** : 1568-1572 .
163. Osborn , M . J . , and H . C . P . Wu . 1980 . Proteins of the outer membrane of gram-negative bacteria . *Ann. Rev. Microbiol.* **34** : 369-422.
 164. Overath , P . , and H . Trauble . 1973 . Phase transitions in cell membranes and lipids of *Escherichia coli*. Detection by fluorescent probes light scattering and dilatometry . *Biochemistry* . **12** : 2625-2634.
 165. Overath , P . , M . Brenner , T . Gulik-Krywicki , E . Schechter , and L . Letellier. 1975 . Lipid phase transitions in cytoplasmic and outer membranes of *Escherichia coli*. *Biochim. Biophys. Acta* . **389** : 358-369 .
 166. Page , W . J . 1982 . Optimal conditions for induction of competence in nitrogen fixing *Azotobacter vinelandii* . *Can. J. Microbiol.* **28** : 389-397 .
 167. Pakula , R . , and W . Walczak . 1963 . On the nature of competence of transformable *Streptococci* . *J. Genet. Microbiol.* **31** : 125-133 .
 168. Papahadjopoulos , D . , W . J . Vail , C . Newton , S . Nir , K . Jacobson , R . Lazo , and G . Poste . 1976 . Studies on membrane fusion. III . The role of calcium induced phase changes . *Biochim. Biophys. Acta* . **465** : 579-595 .
 169. Perroud , B . and D . Le Rudulier . 1985 . Glycine betaine transport in *Escherichia coli* : Osmotic modulation . *J. Bacteriol.* **161** : 393-401 .
-

170. Picksley , S . M . , P . V . Attfield , and R . G . Lloyd .1984a. Repair of DNA double-strand breaks in *Escherichia coli* K-12 requires a functional *recN* product . *Mol. Gen. Genet.* **195** : 267-274 .
171. Piechowska , M . , and M . S . Fox . 1971. Fate of transforming deoxyribonucleate in *Bacillus subtilis* . *J. Bacteriol.* **108** : 680-689 .
172. Pifer , M . L . 1986 . Plasmid establishment in competent *Haemophilus influenzae* occurs by illegitimate transformation . *J. Bacteriol.* **168** : 683-687 .
173. Pilarski , L . M . and J . B . Egan , 1973 . Role of DNA topology in transcription of coliphage λ *in vivo* . II . DNA topology protects the templates from exonuclease attack . *J. Mol. Biol.* **76** : 257-266 .
174. Polsinelli , M . , and S . Barlati . 1967 . Effect of periodate on competence in *Bacillus subtilis* . *J. Gen. Microbiol.* **49** : 267-275 .
175. Raina , J . L . , and A . W . Ravin . 1981 . Switches in macromolecular synthesis during induction of competence for transformation of *Streptococcus sanguis*. *Proc. Natl. Acad. Sci. USA* . **77** : 6062-6066.
176. Radding , C . M . 1978 . Genetic recombination : Strand transfer and mismatch repair . *Ann. Rev. Biochem.* **47** : 847-880 .
177. Radding , C . M . 1981. Recombination activities of *E. coli* *recA* protein . *Cell* **25** : 3-4 .
178. Ranhand , J . M . , and H . C . Lichstein . 1966 . Periodate inhibition of transformation and competence development in *Haemophilus influenzae*

- J. Bacteriol.* **92** : 956-959 .
179. Ranhand , J . M . , and H . C . Lichstein . 1969 . Effect of selected antibiotics and other inhibitors on competence development in *Haemophilus influenzae* . *J. Gen. Microbiol.* **55** : 37 .
180. Ranhand , J . M . 1969 . Competence in *Haemophilus influenzae* . A role for inosine and lactate in the primary cell-deoxyribonucleic acid attachment reaction . *J. Gen. Microbiol.* **57** : 257-262 .
181. Ranhand , J . M . 1980 . Interactions of competent *Streptococcus sanguis* (wicky) cells with native or denatured , homologous or heterologous deoxyribonucleic acids . *J. Bacteriol.* **142** : 568-580 .
182. Reijnders , B . , W . P . M . Hoekstra , J . E . N . Bergmans , and I . M . van Die . 1979 . Transformation in *Escherichia coli* . S . W . Glover , and L . O Butler (eds.) Transformation 1978 . pp. 113-121 . Cotswold Press , Oxford .
183. Reusch , R . N . , T . W . Hiske , and H . L . Sadoff . 1986 . D-(-)-poly-b-hydroxybutyrate membrane structure and its relationship to genetic transformability in *Escherichia coli* . *J. Bacteriol.* **168** : 553-562 .
184. Reusch , R . N . , and H . L . Sadoff . 1983 . D-(-)-poly-b-hydroxybutyrate in membranes of genetically competent bacteria . *J. Bacteriol.* **156** : 778 -788 .
185. Rhoads , D . B . , F . B . Waters , and W . Epstein . 1975 . Cation transport

- in *Escherichia coli*. VIII . Potassium transport mutants . *J. Gen. Physiol.* **67** : 325-341 .
186. Riva , S . , and M . Polcinelli . 1968 . Relationship between competence for transfection and for transformation . *J. Virol.* **2** : 587-593 .
187. Rosen , B . P . , and J . S . McClees . 1974 . Active transport of calcium in inverted membranes of *Escherichia coli* . *Proc. Natl. Acad. Sci. USA* . **71** : 5042-5046 .
188. Sabelnikov , A . G . , S . J . Dityakin , and B . N . Ilyashenko . 1973 . About the role of protamine in transfection . *Biochim. Biophys. Acta* **299** : 492-495 .
189. Sabelnikov , A . G . , A . V . Aydeeva , and B . N . Ilyashenko . 1975 . Enhanced uptake of donor DNA by Ca^{2+} -treated *Escherichia coli* cells . *Mol. Gen. Genet.* **138** : 351-358 .
190. Sabelnikov , A . G . , and I . V . Domaradsky . 1979 . Proton conductor vs. cold in induction of Ca^{2+} -dependent competence in *Escherichia coli* . *Mol. Gen. Genet.* **172** : 313-317 .
191. Sabelnikov , A . G . , and I . V . Domaradsky . 1981 . Effect of metabolic inhibitors on entry of exogenous deoxyribonucleic acid into Ca^{2+} -treated *Escherichia coli* cells . *J. Bacteriol.* **146** : 435-443 .
192. Sabelnikov , A . G . , I . V . Gordienko , and B . N . Ilyashenko . 1984 . Can Ca^{2+} -dependent competence be repeatedly induced in the same

- Escherichia coli* cells ? *Mol. Gen. Genet.* **193** : 538-542 .
193. Salmond , P . G . C . , J . C . D . Hinton , and M . C . M . Perombelon .
1985 . Efficient transformation of *Erwinia carotovora* subsp. *carotovora*
and *E. carotovora* subsp. *atroseptica* . *J. Bacteriol.* **161** : 786-788 .
 194. Saito , H . , and H . Uchida . 1978 . Organization and expression of
the *dnaJ* and *dnaK* genes of *Escherichia coli* K-12 . *Mol. Gen.
Genet.* **164** : 1-8 .
 195. Sancar , A . , C . Stachelek , W . Konigsberg , and W . D . Rupp . 1980 .
Sequences of the *recA* gene and protein . *Proc. Natl. Acad. Sci.
USA* . **77** : 2611-2615 .
 196. Saunders , C . W . , and W . R . Guild . 1981 . Monomer plasmid DNA
transforms *Streptococcus pneumoniae* . *Mol. Gen. Genet.* **181** :
57-62 .
 197. Saunders , J . R . , A . Ball , M . G . M . Brown , G . O . Humphreys , and
C Edwards 1987 . Cell volume changes correlate with efficiency of
transformation of *Escherichia coli* with plasmid DNA . *F. E. M. S.
Microbiology Letters* **42** : 125-128 .
 198. Scher , B . , and D . Dubnau . 1976 . Purification and properties of a
magnesium stimulated endonuclease from *Bacillus subtilis* . *J.
Bacteriol.* **126** : 429-438 .
 199. Scoocca , J . J . , R . L . Poland , and K . C . Zoon . 1974 . Specificity in
deoxyribonucleic acid uptake by transformable *Haemophilus influenzae*

- J. Bacteriol.* **118** : 369-373 .
200. Seto , H . , and A . Tomasz . 1974. Early stages in DNA binding and uptake during genetic transformation in Pneumococci . *Proc. Natl. Acad. Sci. USA* **71** : 1493-1498 .
201. Seto , H . , and A . Tomasz . 1976 . Calcium-requiring step in the uptake of deoxyribonucleic acid molecules through the surface of competent pneumococci . *J. Bacteriol.* **126** : 1113-1118 .
202. Shibata , T . , R . P . Cunningham , C . DasGupta , and C . Radding . 1979 . Homologous pairing in genetic recombination : Complexes of *recA* protein and DNA . *Proc. Natl. Acad. Sci. USA* . **76** : 5100-5104.
203. Shimshick , H . J . , and H . M . McConnell . 1973 . Lateral phase separation in phospholipid membranes . *Biochemistry* . **12** : 2351-2360.
204. Silver , S . , K . Toth , and H . Scribner . 1975 . Facillitated transport of calcium by cells and subcellular membranes of *B. subtilis* and *E. coli* . *J. Bacteriol.* **122** : 880-885 .
205. Sisco , K . L . , and H . O . Smith . 1979 . Sequence-specific DNA uptake in *Haemophilus* transformation . *Proc. Natl. Acad. Sci. USA* **76** : 972-976 .
206. Singh , R . N . , and M . P . Pitale . 1967 . Enrichment of *Bacillus subtilis* transformants by zonal centrifugation . *Nature* (London) **213** : 1262-1263 .
207. Squires , C . H . , M . DeFelice , S . R . Wessler , and J . M . Calvo , 1981 .

- Physical characterization of the *hly* HI operon of *Escherichia coli* K-12 .
J. Bacteriol. **147** : 797-804 .
208. Sneath , P . H . A . , and J . Lederberg .1961 . Inhibition by periodate of mating in *Escherichia coli* K-12 . *Proc. Natl. Acad. Sci. USA* **47** : 86-90 .
209. Smith , H . O . , D . B . Danner , and R . A . Deich . 1981 . Genetic transformation *Ann. Rev. Biochem.* **50** : 41-68
210. Smith , H . , K . Wiersma , and G . Venema , and S . Bron . 1984 . Transformation in *Bacillus subtilis* : A 75,000-dalton protein complex is involved in binding and entry of donor DNA . *J. Bacteriol.* **157** : 733-738 .
211. Smith , H . , W . deVos , and S . Bron . 1983a . Transformation in *Bacillus subtilis* : Properties of DNA binding-deficient mutants . *J. Bacteriol.* **153** : 12-20 .
212. Smith , H . , H . K . Wiersma , S . Bron , and G . Venema . 1983b . Transformation in *Bacillus subtilis* : Purification and partial characterization of a membrane-bound DNA-binding protein . *J. Bacteriol.* **156** : 101-108 .
213. Smith , H . , K . Wiersma , and G . Venema , and S . Bron .1985 : Transformation in *Bacillus subtilis* : Further characterization of a 75,000-dalton protein complex involved in binding and entry of donor DNA . *J. Bacteriol.* **164** : 201-206 .

214. Smith , H . O . , and M . E . Khan . 1984 . Transformation in *Haemophilus*
A problem in membrane biology . *J. Membr. Biol.* **81** : 89-103 .
215. Spencer , H . T . , and R . M . Herriott . 1965 . Development of
competence of *Haemophilus influenzae* . *J. Bacteriol.* **90** : 911-920
216. Stewart , J . G . , and C . A . Carlson . 1986 . The biology of natural
transformation . *Ann. Rev. Microbiol.* **40** : 211-235 .
217. Streips , U . N . , and F . E . Young . 1971 . Mode of action of the
competence-inducing factor of *Bacillus stearothermophilus* . *J.*
Bacteriol. **106** : 868-875 .
218. Strike , P . , G . O . Humphreys , and R . S . Roberts . 1979 . Nature of
transforming deoxyribonucleic acid in calcium-treated *Escherichia coli* .
J. Bacteriol. **138** : 1033-1035 .
219. Stuy , J . H . 1979 . Plasmid transfer in *Haemophilus influenzae* . *J.*
Bacteriol. **139** : 520-529 .
220. Stuy , J . H . 1980b . Mechanism of *Haemophilus influenzae* transfection
by single and double prophage deoxyribonucleic acid . *J. Bacteriol.*
144 : 1003-1008 .
221. Stuy , J . H . 1985 . Transfer of genetic information within a colony of
Haemophilus influenzae . *J. Bacteriol.* **162** : 1-4 .
222. Summers , D . K . , and D . J . Sherratt . 1984 . Multimerization of high
copy number plasmids causes instability : ColE1 encodes a determinant
essential for plasmid monomerization and stability . *Cell.* **36** : 1097-

1103.

223. Sunshine , M . , M . Feiss , J . Stuart , and J . Yochem . 1977 . A new host gene (*groPC*) necessary for lambda DNA replication . *Mol. Gen. Genet.* **151** : 27-34 .
224. Tabor , H . 1961 . The stabilization of *Bacillus subtilis* transforming principle by spermine . *Biochem. Biophys. Res. Comm.* **4** : 228-231
225. Tabor , H . , and C . W. Tabor . 1972 . Biosynthesis and metabolism of 1,4-diaminobutane , spermidine , spermine and related amines . *Adv. Enzymol* **36** : 203-268 .
226. Tabor , C . W. , and H . Tabor . 1985 . Polyamines in microorganisms . *Microbiol. Rev.* **49** : 81-99 .
227. Taketo , A . , and S . Kuno . 1969a . Sensitivity of *Escherichia coli* to viral nucleic acid . I . Effect of lysozyme , EDTA , Penicillin and osmotic shock treatment . *J. Biochem.* **65** : 361-368 .
228. Taketo , A . 1972 . Sensitivity of *Escherichia coli* to viral nucleic acid . V . Competence of calcium-treated cells . *J. Biochem.* **72** : 973-979 .
229. Taketo , A . 1974 . Sensitivity of *Escherichia coli* to viral nucleic acid . VIII . Idiosyncrasy of Ca^{2+} -dependent competence for DNA . *J. Biochem* **75** : 895-904 .
230. Taketo , A . 1977 . Sensitivity of *Escherichia coli* to viral nucleic acid . XII . Ca^{2+} - or Ba^{2+} - facilitated transfection of cell envelope mutants . *Z. Naturforsch* **32c** : 429-433 .

231. Taketo , A . 1975 . Sensitivity of *Escherichia coli* to viral nucleic acid .
X. Ba²⁺- induced competence for transfecting DNA . *Z . Naturforsch* 30:
520-522 .
232. Taketo , A . 1978 . Effect of mutations in surface lipopolysaccharide , or
outer membrane protein of *Escherichia coli* C on transfecting
competence for microvirid phage DNA . *J. Gen. Appl. Mikrobiol.*
24 : 51-58 .
233. Tanner , D . , and M . Oishi . 1971. The effect of bacteriophage T4
infection on an ATP-dependent deoxyribonuclease in *Escherichia coli*.
Biochim. Biophys. Acta **228** : 767-769 .
234. Taylor , A . F . , and G . R . Smith . 1980 . Unwinding and rewinding of
DNA by the *recBC* enzyme . *Cell* **22** : 447-459 .
235. Taylor , A . F . , and G . R . Smith . 1985 . Substrate specificity of the
DNA unwinding activity of the *recBC* enzyme of *Escherichia coli*. *J. Mo.
Biol.* **185** : 431-443 .
236. Telander-Muskavitch , K . M . , and S . Linn . 1981 . RecBC-like
Enzymes : Exonuclease V. deoxyribonuclease . P. D . Boyer (ed.) pp.
233-250 The Enzymes vol . 14 , Academic Press Inc . N . Y .
237. Tigrari , S . , and B . E . B. Moseley . 1980 . Transformation in
Mikrococcus radiodurans : Measurement of various parameters and
evidence for multiple independently segregating genomes per cell . *J.
Gen. Mikrobiol.* **119** : 287-296 .

238. Tomasz , A . , and R . D . Hotchkiss . 1964 . Regulation of the transformability of pneumococcal cultures by a macromolecular cell product . *Proc. Natl. Acad. Sci. USA* . **51** : 480-487 .
 239. Tomasz , A . , and S . M . Beiser . 1965 . Relationship between the competence antigen and the competence-activator substance in Pneumococci . *J. Bacteriol.* **90** : 1226-1232 .
 240. Tomasz , A . , and J . L . Mosser . 1966 . On the nature of pneumococcal activator substance . *Proc. Natl. Acad. Sci. USA* . **55** : 58-66 .
 241. Tomasz , A . 1969 . Some aspects of the competent state in genetic transformation . *Annu. Rev. Genet.* **3** : 217-232 .
 242. Tomasz , A . 1970 . Cellular metabolism in genetic transformation of pneumococci : requirement for protein synthesis during induction of competence . *J. Bacteriol.* **101** : 860-871 .
 243. Tomasz , A . 1973 . Bacterial Transformation . L . J . Archer (ed .) pp. 81-88 . Academic Press Inc . N . Y .
 244. Tomasz , A . , and D . Horne . 1985 . Competence-specific autolysis in *Streptococcus sanguis* . *J. Gen. Microbiol.* **131** : 533-541 .
 245. Tsujibo , H . , and B . P . Rosen . 1983 . Energetics of calcium efflux from cells of *Escherichia coli* . *J. Bacteriol.* **154** : 854-858 .
 246. Unger , R . and A . J . Clark . 1972 . Interaction of the recombination pathways of bacteriophage I and its host *Escherichia coli* K-12 : Effects on λ recombination . *J. Mol. Biol.* **70** : 539 .
-

247. van Alphen, L., B. Lugtenberg, and R. van Bortel. 1977.
Architecture of the outer membrane of *Escherichia coli* K-12. II. Freeze fracture morphology of wild-type and mutant strains. *Biochim. Biophys. Acta* **466** (2): 257-268.
248. van Alphen, L., A. Verkleij, J. Leunissen-Bijvelt, and B. Lugtenberg
1978. Architecture of the outer membrane of *Escherichia coli*. III
Protein-lipopolysaccharide complexes in intramembranous particles.
J. Bacteriol. **134**: 1089-1098.
249. van Die, I. M., H. E. N. Bergmans, and W. P. M. Hoekstra. 1983.
Transformation in *Escherichia coli*: Studies on the role of the heat
shock in induction of competence. *J. Gen. Microbiol.* **129**: 663-670.
250. Venema, G. 1979. Bacterial transformations. *Adv. Microb. Phys.*
19: 245-331
251. Verkleij, A. 1975. "Architecture of biological and
artificial membranes as visualized by freeze etching". PhD Thesis.
State University of Utrecht. Holland.
252. Vijayakumar, M. N. and D. A. Morrison. 1986. Localization of
competence-induced proteins in *Streptococcus pneumoniae*. *J.*
Bacteriol. **165**: 689-695.
253. Villarejo, M., J. L. Davis, and S. Granett. 1983. Osmoregulation
of alkaline phosphatase synthesis in *Escherichia coli* K-12. *J.*
Bacteriol. **156**: 975-978.
254. Volvis, G. F., and G. Buttin. 1970. An ATP-dependent

- deoxyribonuclease from *Diplococcus pneumoniae* . II Evidence for its involvement in bacterial recombination . *Biochem. Biophys. Acta* **224** : 42-54 .
255. Wackernagel , W . 1973 . Genetic transformation in *E. coli* : The inhibitory role of the *recBC* DNase . *Biochem. Biophys. Res. Comm* **51** : 306-311 .
256. Weston , A . , G . O . Humphreys , M . G . M . Brown , and J . R . Saunders 1979. Simultaneous transformation of *Escherichia coli* by pairs of compatible and incompatible plasmid DNA molecules . *Mol. Gen. Genet.* **172** : 113-118 .
257. Weston , A . , M . G . M . Brown , H . R . Perkins , J . R . Saunders , and G . O . Humphreys . 1981. Transformation of *Escherichia coli* with plasmid deoxyribonucleic acid : Calcium binding of deoxyribonucleic acid to whole cells and to isolated membrane fractions . *J. Bacteriol.* **145** : 780-787 .
258. Wickner, S . H . 1978 . DNA replication proteins of *Escherichia coli* and phage λ . Cold Spring Harbor Symp. Quant . Biol . **43** : 303-310 .
259. Willets , N . S . , and D . W . Mount . 1969 . Genetic analysis of recombination-deficient mutants of *Escherichia coli* K-12 carrying *rec* mutations cotransducible with *thyA* . *J. Bacteriol.* **100** : 923-934 .
260. Wise , E . M . , S . P . Alexander , and M . Powers . 1973 . Adenosine 3'

- 5'-cyclic monophosphate as a regulator of bacterial transformation .
Proc. Natl. Acad. Sci USA **70** : 471-475 .
261. Yamamoto , K . , B . Alberts , R . Benzinger , L . Lawhorne , and G .
 Treiber . 1970. Rapid bacteriophage sedimentation in the presence of
 polyethylene glycol and its application to large -scale virus purification
Virology . **40** : 734-744 .
262. Yasbin , R . E . , M . J . Tevethia , G . A . Wilson , and F . E . Young . 1973
 Bacterial Transformation . L . J . Archer (ed .) pp. 2-36 . Acad . Press Inc
 N . Y .
263. Young , F . E . , and R . L . Sinsheimer . 1967 . Vegetative
 bacteriophage lambda DNA . I . Infectivity in a spheroplast assay . *J.*
Mol . Biol. **30** : 147-164 .
264. Young , F . E . , and J . Spizizen . 1963 a . Biochemical aspects in
 competence in the *Bacillus subtilis* transformation system . II . Autolytic
 enzyme activity of cell walls . *J. Biol. Chem.* **238** : 3126-3130 .
265. Zoon , J . , M . Haberstat , and J . J . Scocca . 1976 . Synthesis of
 envelope polypeptides by *Haemophilus influenzae* during development
 of competence for genetic transformation . *J. Bacteriol.* **127** : 545-554 .