

Studies on Transformation of *Escherichia coli* with Plasmids

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Factors that affect the probability of genetic transformation of *Escherichia coli* by plasmids have been evaluated. A set of conditions is described under which about one in every 400 plasmid molecules produces a transformed cell. These conditions include cell growth in medium containing elevated levels of Mg^{2+} , and incubation of the cells at 0°C in a solution of Mn^{2+} , Ca^{2+} , Rb^{+} or K^{+} , dimethyl sulfoxide, dithiothreitol, and hexamine cobalt (III). Transformation efficiency declines linearly with increasing plasmid size. Relaxed and supercoiled plasmids transform with similar probabilities. Non-transforming DNAs compete consistent with mass. No significant variation is observed between competing DNAs of different source, complexity, length or form. Competition with both transforming and non-transforming plasmids indicates that each cell is capable of taking up many DNA molecules, and that the establishment of a transformation event is neither helped nor hindered significantly by the presence of multiple plasmids.

1. Introduction

Both gram-positive and gram-negative bacteria can take up and stably establish exogenous DNA. Many of their characteristics in genetic transformation, however, are different (Hotchkiss & Gabor, 1970; Lewin, 1977; Smith *et al.*, 1981). For example, gram-positive bacteria interact with double-stranded DNA but transfer only one strand into the cell, whereas gram-negative bacteria preferentially interact with and transfer double-stranded DNA. However, there are features in common to both. One is the existence of a transitory state of competence for transformation, which is generally related both to the conditions of growth and to the circumstances under which the cells and DNA are combined. Another is that divalent cations play important (and often essential) roles in the early stages of DNA uptake.

DNA transfer into *Escherichia coli* was first demonstrated by Mandel & Higa (1970), who reported that bacteriophage DNAs could be transfected into cells with the consequent appearance of infectious centers of virus. Transfection occurred when the cells and DNA were combined in the presence of 50 mM- Ca^{2+}

at 0°C, and subjected to a brief heat pulse at 37 to 42°C. These conditions were shown to induce a general state of competence for DNA uptake through their subsequent application to genetic transformation (Cosloy & Oishi, 1973a), in which linear prototrophic *E. coli* DNA was transferred into auxotrophic strains to restore mutant alleles, as well as to plasmid transformation (Cohen *et al.*, 1972), whereby plasmids carrying antibiotic resistance genes were transferred into *E. coli*, and stably established as multicopy episomes.

The necessary conditions for DNA transfer into *E. coli* have been examined in further detail using each of these three related criteria: bacteriophage transfection (Taketo, 1972, 1974, 1975; Taketo & Kuno, 1974); genetic transformation (Cosloy & Oishi, 1973b; Sabelnikov *et al.*, 1975; Bergmans *et al.*, 1981); and plasmid transformation (Lederberg & Cohen, 1974; Kretschmer *et al.*, 1975; Norgard *et al.*, 1978; Kushner, 1978; Strike *et al.*, 1979; Weston *et al.*, 1979; Sabelnikov & Domaradsky, 1979; Dagert & Ehrlich, 1979; Bergmans *et al.*, 1980; Jones *et al.*, 1981).

The consensus that develops from these investigations is that *E. coli* cells and DNA interact productively in an environment of calcium ions and low temperature (0 to 5°C), and that a heat pulse is important (though not strictly required). Several other factors have been shown to stimulate the efficiency of DNA transfer into *E. coli*: combinations of Ca^{2+} and Mg^{2+} (Taketo & Kuno, 1974; Wensink *et al.*, 1974), Ca^{2+} and Mn^{2+} (Enea *et al.*, 1975; A. Bothwell, unpublished observations), and Ca^{2+} , Rb^{+} and dimethyl sulfoxide (Kushner, 1978); the substitution of other alkali earth metals for Ca^{2+} (Taketo, 1975); and extended incubation of Ca^{2+} -treated cells at 0°C (Taketo, 1972; Dagert & Ehrlich, 1979).

The efficiency with which plasmids transform *E. coli* has remained low and the process obscure. The purpose of this investigation was to improve transformation efficiency and to further characterize the transformation process. A set of conditions is described in which transformation efficiencies are enhanced 100 to 1000 times over those obtained by treatment with CaCl_2 . In essence, these conditions are cell growth in elevated levels of Mg^{2+} , and subsequent combination of cells and DNA in the presence of Mn^{2+} , Ca^{2+} , Rb^{+} or K^{+} , dimethyl sulfoxide, dithiothreitol, and hexamine cobalt (III) chloride. Plasmid transformation under these conditions has been characterized with regard to plasmid size and superhelicity, the effects of competing DNAs (both transformable and non-transformable), linearity (dose response), cell growth conditions, and the applicability to various strains of *E. coli* K12.

Transformation of *E. coli* with plasmids carrying antibiotic resistance genes is defined by the appearance of isolated colonies on selective plates. The transformation event can be divided into two general phases, uptake of DNA across the cell envelope, and establishment of that DNA as a stable genetic element in the cell. Two criteria are used in evaluating transformation. One is the probability that a plasmid molecule will produce a transformed cell, given either in natural units (P_p) or expressed as colonies formed per picogram or microgram of plasmid DNA (the transformation efficiency, XFE). The second is the probability (F_c) that a viable cell will become transformed. The transformation

probability P_p has been the primary criterion employed to assess the effects of difference conditions since, in general, the relevant parameter in a transformation is the efficiency with which a single plasmid can be introduced into and established within a cell.

2. Materials and Methods

(a) *Strains*

The strains used in this study are listed and described in Table 4. In general, strains were stored at -70°C . A few fresh colonies were innoculated into 5 ml of SOB medium (see section (c), below) and incubated with agitation until the cell density was 10^8 to 2×10^8 /ml. The culture was diluted 1:1 into 40% glycerol/60% SOB medium, chilled on ice, and portions taken into serially numbered screw-cap polypropylene tubes (e.g. Nunc). The cells were kept on ice for 10 min and then flash frozen in solid CO_2 /ethanol, and placed at -70°C . On the day before a transformation, a tube was removed from the freezer, a clump of cells scraped up with a sterile tip, the tube replaced immediately (without thawing), and the clump of cells placed on an LM (see section (c), below) plate. Once melted, the cells were spread to isolate single cells and placed at 37°C to develop colonies. Each tube was used 5 to 10 times and discarded, after which the next tube was used. A new frozen stock should be made up yearly.

DH1 was constructed as follows. *thyA* was introduced into MM294 by selection with $50\text{ }\mu\text{g}$ trimethoprim/ml. This strain (MMT) was mated with KL16-99 (an Hfr for *thyA*⁺ *recA*⁻; Low, 1968) and the cells plated on minimal glycerol plates, where neither parent will grow. Colonies that developed on this medium were restreaked and assayed for u.v.† sensitivity. A u.v.-sensitive isolate was designated DH2. The *gyrA* mutation was then introduced by selection for growth on $100\text{ }\mu\text{g}$ naladixic acid/ml, and this *recA* derivative of MM294 was designated DH1. Studies using concatenated plasmids, which do not resolve to monomers in DH1, indicate that this strain is deficient in homologous recombination. DH1 is available from the *E. coli* Genetic Stock Center, Yale University (CGSC no. 6040), and from the American Type Culture Collection, Rockville, MD (ATCC no. 33849).

F' derivatives of DH1 (DH20 and DH21) were constructed by mating DH1 with strain X90 (*ara*, ∇ *lac-pro*, *nalA*, *argEam*, *rif*^R, *thi-1*) carrying either F' *lacI*^q (Muller-Hill *et al.*, 1968) or F' *lacI*^{sq} (Miller *et al.*, 1970) and plating the culture on minimal glycerol Xgal plates, which select against the donor. DH1 and DH1/F' are distinguished by the slower growth rate of the F' and a colony phenotype of white colonies with blue centers on non-inducing Xgal plates, a characteristic of these F' *lacI* strains but not of their F⁻ parents. Neither DH20 or DH21 cure their F' with an appreciable frequency in liquid culture; large white colonies are observed only rarely in streaks and avoided when picking colonies for transformations.

(b) *DNAs*

Plasmids were banded on CsCl/EtBr density gradients and passed over Biogel A50f columns (to remove transfer RNA). DNA concentrations were determined using a Gilford u.v. spectrophotometer and verified by comparison with known pBR standards on agarose gels.

Relaxed DNAs were prepared by treating supercoiled DNAs with calf thymus topoisomerase I (a gift from J. Wang): $2\text{ }\mu\text{g}$ of each DNA were incubated for 30 min at 20°C in the presence of topoisomerase I in a buffer comprised of 10 mM-Tris (pH 8), 0.2 M-NaCl, 0.1 mM-EDTA, and $50\text{ }\mu\text{g}$ bovine serum albumin/ml. DNAs were then extracted twice with phenol, twice with chloroform, and precipitated with isopropanol twice. The relaxed DNAs

† Abbreviations used: u.v., ultraviolet light; EtBr, ethidium bromide; MES, morpholinoethane sulfonic acid; H₂CoCl₃, hexamine cobalt(III) chloride; DMSO, dimethyl sulfoxide; kb, 10^3 bases or base-pairs as appropriate; bp, base-pairs; DTT, dithiothreitol.

were resuspended and analyzed on gels to assess relaxation, and concentrations determined both from gels and by absorbance at 260 nm and 280 nm using a u.v. spectrophotometer.

High molecular weight DH1 *E. coli* DNA was prepared as follows. A saturated culture of DH1 in SOB medium was pelleted, resuspended in TE buffer (see below), made 0.5% (w/v) in sodium dodecyl sulfate, and incubated for 10 min. Pronase was added to 100 µg/ml, and the suspension incubated for 12 h at 37°C, followed by extraction with phenol (twice), CHCl₃ (twice), and dialysis against TE buffer (12 h at 4°C). RNase was added to 100 µg/ml and the solution incubated for 2 h at 37°C, followed by a second 2 h incubation in the presence of 10 µg Pronase/ml. The DNA was extracted with phenol (twice), CHCl₃ (twice), and dialyzed extensively against TE buffer at 4°C.

Hinf-restricted DNAs were prepared by digestion with *Hinf* under conditions recommended by the vendor (N.E. Biolabs), and then extracted with phenol and chloroform, precipitated in 2 M-ammonium acetate with 2 vol. isopropanol, and reprecipitated in 0.3 M-ammonium acetate with 2 vol. isopropanol.

DNAs were stored at 4°C and diluted for transformations into 0.5 × TE buffer (5 mM-Tris (pH 7.4), 0.5 mM-EDTA). All experiments were conducted in accord with the U.S. guidelines governing recombinant DNA research.

(c) Media and plates

All plates were LM: 1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract, 10 mM-NaCl, 10 mM-MgSO₄·7H₂O, 1.5% (w/v) Bacto agar. For tetracycline plates, the Mg²⁺ was omitted. Tryptone, yeast extract and agar were from Difco Labs. All antibiotics were used at 35 µg/ml, except for tetracycline, which was used at 17 µg/ml.

SOB medium is 2% (w/v) Bacto Tryptone, 0.5% (w/v) yeast extract, 10 mM-NaCl, 2.5 mM-KCl, 10 mM-MgCl₂, 10 mM-MgSO₄. SOB medium was prepared without Mg²⁺ and autoclaved. A 2M stock of Mg²⁺ (1 M-MgCl₂·6H₂O + 1 M-MgSO₄·7H₂O, sterile filtered) was used to make the medium 20 mM in Mg²⁺, after which it was sterile filtered through a prerinsed 0.22 µm filter unit. The final pH was 6.8 to 7.0.

SOC medium is SOB medium containing 20 mM-glucose, and was prepared similarly; before filtration, the medium was made 20 mM in glucose using a sterile filtered 2 M stock. The water used for these media was the purest available.

(d) Chemicals

The chemicals used in this investigation were obtained from the following sources: 2-*N*-morpholinoethane sulfonic acid, Research Organics, Cleveland, Ohio (no. 0113M), or Sigma (no. M8250); rubidium chloride, Alpha Products, Danvers, MA. (no. 88688) or MCB (no. RX185); manganese chloride·4H₂O, MCB (no. MX185); calcium chloride·2H₂O, Fisher (no. C79); hexamine cobalt (III) chloride, Alpha Products (no. 23144), Fluka (no. 52740), Aldrich (no. 20,309-2), or Kodak (no. 8253); dithiothreitol, Calbiochem (no. 233155); dimethyl sulfoxide, specpure glass distilled, Alpha (no. 13778), MCB (no. MX1456), or Mallinkrodt (no. 5507); glycerol, glass distilled, BRL (no. 5514), or Alpha (no. 13797); potassium and magnesium salts, Mallinkrodt; mercaptoethanol, Eastman Organics (no. 4196).

(e) Solutions and buffers

TFB (standard information buffer) is 10 mM-K-MES (pH 6.20), 100 mM-RbCl, 45 mM-MnCl₂·4H₂O, 10 mM-CaCl₂·2H₂O, 3 mM-HACoCl₃. 1 M-MES is adjusted to pH 6.3 using KOH, sterile filtered, and stored at -20°C. All salts are added as solids. The solution is sterile filtered through a prerinsed 0.22 µm filter unit and stored at 4°C. TFB is stable at 4°C for >1 year (final pH 6.15 ± 0.10). KCl may be substituted for RbCl using DH1 and its relatives (other strains have not been tested).

FSB (transformation buffer for frozen storage of competent cells) is 10 mM-potassium acetate, 100 mM-KCl, 45 mM-MnCl₂·4H₂O, 10 mM-CaCl₂·2H₂O, 3 mM-HACoCl₃, 10% redistilled glycerol. 1 M-potassium acetate is adjusted to pH 7.0, sterile filtered, and stored frozen. All salts are added as solids. The pH of the complete solution is adjusted to 6.4 with

0.1 M-HCl, and the solution is sterile filtered and stored at 4°C; the pH drifts downward to a final value of 6.1 to 6.2 and then stabilizes. RbCl may be required for some strains.

DMSO is stored as 550- μ l portions in 0.5 ml Eppendorf polypropylene tubes. A sealed bottle of glass-distilled DMSO is opened and the entire contents portioned out and stored at -20°C. A single portion is thawed and used on a given day and then discarded. (Oxidation products of DMSO are very inhibitory.)

The dithiothreitol solution is 2.25 M-DTT, 40 mM-potassium acetate (pH 6.0). It is sterile filtered, placed into 0.5 ml polypropylene tubes, and stored at -20°C. A tube is thawed, used, and promptly refrozen. The stock solution of 2.25 M-dithiothreitol may be substituted with 750 mM-2-mercaptoethanol, 10 mM-K-MES (pH 6.2) (provisional).

The water used was the purest available for all solutions and growth media. Water purified by reverse osmosis (Millipore Milli-Q) has been used exclusively in this study.

(f) *Standard transformation*

Colonies were picked off a fresh streak from the frozen stock of cells (one 2.5 mm diam. colony/10 to 15 ml) and dispersed in 1 ml of SOB medium by moderate vortexing. This was used to inoculate a prerinsed flask of SOB medium: 10 to 30 ml in a 300-ml flask, 30 to 100 ml in a 1000-ml flask, etc. The culture was incubated at 37°C, 275 revs/min, until the cell density was 4×10^7 to 7×10^7 /ml (absorbance at 550 nm = 0.45 to 0.55 for DH1; in about 2 to 2.5 h). The cells were collected into 50-ml polypropylene tubes (e.g. Falcon 2070), placed on ice for 10 to 15 min, and pelleted at 2500 revs/min for 12 min at 4°C. The cells were resuspended in 1/3 vol. TFB by gentle vortexing, placed on ice for 10 to 15 min, and pelleted again at 2500 revs/min for 10 min at 4°C. The pellet was resuspended in TFB at 1/12.5 of the original volume of cells (2.5 ml of the culture is concentrated into 200 μ l, one discrete transformation). Fresh DMSO was added to 3.5% (7 μ l/200 μ l), swirled, and left on ice for 5 min. DTT was added to 75 mM (7 μ l of stock/200 μ l), swirled, and left on ice for 10 min. Another equal portion of DMSO was added, and the cells incubated for 5 min on ice. Samples (210 μ l) were then placed into chilled 17 mm \times 100 mm polypropylene tubes (Falcon 2059). DNA was added (in <10 μ l), and the mixture swirled and incubated on ice for 30 min. The mixture was heat-pulsed without agitation at 42°C for 90 s, and placed on ice for 1 to 2 min. Then 800 μ l of SOC medium (~20°C) was added and the tubes incubated at 37°C, 225 revs/min, for 1 h. An appropriate fraction of the culture was pipetted in a pool of SOB medium (100 to 200 μ l) on an LM plate (with appropriate antibiotic), and spread gently and minimally using a bent (L-shaped) Pasteur pipette. These plates were incubated at 37°C to establish colonies. If more than 10% of the transformation was to be plated, the cells were washed and concentrated as described in section (k), below.

The heat pulse has been calibrated for 17 mm \times 100 mm polypropylene tubes (Falcon 2059). If other tubes are used, the length of the heat pulse should be recalibrated. For example, a scaled up transformation, in which 25 ml of cells are concentrated into 2 ml, can be performed in a 50-ml polypropylene tube (Falcon 2070). In this case, the optimal time for the heat pulse is 210 s. A scaled up transformation may be concentrated and plated on two 150 mm plates or four 100 mm plates.

(g) *Specifications*

The experimental procedures and sources of chemicals and other materials have been described in considerable detail, in order to allow other investigators to evaluate their results in the event that transformation efficiencies are not in accord with those reported here. Many steps are not critical. For example, some flexibility is possible in the times and forces of centrifugation, as well as in most incubation times (except the heat pulse). However, the culture conditions are important: large flasks used with good surface-to-volume ratios of medium and vigorous agitation (but little foaming) improve subsequent transformation. Virtually all the chemicals and supplies are likely to be readily substituted with those of similar quality from other manufacturers. However, problems have been encountered, such as: soap and organic matter on glassware; surfactants on plastic ware;

impure water; partially oxidized DMSO, and aberrant growth in one brand of yeast extract (M. D. Scott & P. W. J. Rigby, personal communication). Should transformation efficiencies be unacceptably low, it may be very useful to compare materials and procedures with those specified in detail here, in conjunction with application of the controls described in the next section.

(h) Controls

(i) Viable cell counts

Cells were serially diluted twice (10 μ l into 1000 μ l of SOC' medium), 10 μ l was pipetted into a 200- μ l pool of SOB medium on an LM plate, and the pool spread minimally (to give a 10^{-6} dilution). The corresponding density of viable cells should have been 3.5×10^7 to 7×10^7 /ml when the culture was collected, and the cell density at the end of the 60-min incubation in SOC' medium should be 2 to 2.5 times that value, with >80% viability through the procedure (the cells do not divide during the incubation period). If the density of viable cells when collected is low, and inconsistent with optical density, there may be problems with the inoculum or the growth conditions. If the viability is low after the transformation, then tubes or additives may be at fault (see (iii), below).

(ii) XFE

From 10 pg to 1 ng was used in a transformation, plating 1% to 0.01%, respectively (10 colonies = $10^8/\mu$ g, etc.). F_c , 300 to 500 ng pBR was used, plating 10^6 dilution \pm antibiotic (2 to 4% should be transformed). Equal transformation efficiencies were obtained using pBR and DH1 when selecting for either tetracycline or ampicillin resistance.

(iii) Quality of DMSO and DTT

The concentrated cells were divided into separate tubes after the wash, and 4 were carried through the procedure, adding DMSO (twice), dithiothreitol, both, or neither, using 10 pg of pBR. The relative XFE values should approximate those given in Fig. 1. If they do not, then the appropriate compound should be replaced.

(i) Frozen storage of competent cells

Cells were prepared as described above, except that FSB was used. DMSO was added twice and DTT was omitted. The cells were portioned out into 2059 tubes or screw-cap polypropylene tubes (e.g. Nunc), flash-frozen in solid CO_2 /EtOH or liquid N_2 , and placed at -70°C . To use, tubes were removed and thawed in air at 20°C , and, when just liquid, placed on ice for 10 min. DNA was added and the cells heat-pulsed and incubated as described above (XFE: 10^8 to 3×10^8 colonies/ μ g).

(j) Colony transformation

Two to four fresh 2.5 mm diam. colonies were picked carefully off an LM plate (taking no agar) and dispersed in 200 μ l of TFB in a 2059 tube by vortexing gently but thoroughly, and the cells incubated on ice for 15 to 20 min. DMSO, dithiothreitol, and DNA were added as in the standard transformation (XFE 10^8 to 2×10^8 colonies/ μ g).

For the purpose of reintroducing a cloned plasmid into *E. coli*, a few colonies may be dispersed in TFB, incubated on ice for 10 min, combined with DNA, incubated for 10 min, and plated (XFE 10^3 to $10^4/\mu$ g).

(k) Storage of transformed cells

Transformed cells may be stored overnight at 4°C following the 1 h incubation at 37°C in SOC medium. The titer of transformed cells was about 80% after 12 h. For longer storage, the cells should be washed after the incubation. Two ml of SOC medium were added to each tube, and the cells were pelleted at 2000 revs/min for 5 min at 20°C . The pellet was resuspended in SOC medium and placed at 4°C . The titer remains $\sim 90\%$ for several days. For long-term storage, the transformation was diluted 1:1 with 40% glycerol/60% SOB medium, chilled on ice, flash-frozen in solid CO_2 /EtOH, and placed at -70°C . The titer

after thawing was >90%. If an entire transformation is to be put on 1 or 2 plates, the cells should be washed in SOC medium and then resuspended in SOB medium.

3. Results

(a) *Factors influencing plasmid transformation*

The process of identifying conditions that improve transformation probabilities over those achieved by treatment with Ca^{2+} involved two approaches. Initially, factors previously implicated in transformation of *E. coli* were examined in different combinations. A beneficial combination was then used as a basis for examining the effect of other compounds when added to the basic conditions, in order to identify additional stimulants.

An extensive series of experiments examined the effect of various conditions on the transformation efficiency of *E. coli* strain χ 1776 (Curtiss *et al.*, 1977). Compounds were analyzed in different combinations, and the combination of Mn^{2+} , Ca^{2+} , Rb^+ and DMSO was found to be particularly beneficial. The concentration of each compound was optimized, and a set of conditions established that considerably improved the transformation of χ 1776. These conditions are 45 mM- MnCl_2 , 10 mM- CaCl_2 , 100 mM- RbCl , 35 mM-potassium acetate (pH 5.80), 7% (v/v) DMSO, 15% (w/v) sucrose, 0°C, one or two -50°C freeze/thaw cycles, and a 42°C heat pulse.

This set of conditions proved to be inapplicable to a number of other *E. coli* strains. However, the strain MM294 (Meselson & Yuan, 1968) transformed more readily under these conditions than in the standard Ca^{2+} conditions. A *recA* derivative of MM294 was constructed as described in Materials and Methods. This strain, DH1, has been used in subsequent plasmid transformation experiments.

A survey was conducted for other compounds that stimulated the transformation probability (P_p) of DH1, using as a basis the χ 1776 conditions. The stabilizer (sucrose) and the freeze/thaw cycles were eliminated, and potassium acetate was replaced with potassium 2-*N*-morpholinoethane sulfonic acid (K-MES, pH 6.2). Compounds to be tested were added to the transformation buffer at 10 mM concentrations, and assayed in a standard transformation using 10 pg of pBR. About 40 compounds were tested, and two significantly enhanced transformation efficiency: dithiothreitol and hexamine cobalt (III) trichloride. The concentrations of dithiothreitol and HACoCl_3 were optimized, and those of the other factors re-examined. The following set of conditions was established as standard for the subsequent transformation experiments described in this paper: 45 mM- MnCl_2 , 10 mM- CaCl_2 , 100 mM- RbCl , 3 mM- HACoCl_3 , 10 mM-K-MES (pH 6.2), 7% DMSO, 75 mM-dithiothreitol, 0°C, and a 42°C heat pulse. A detailed description of the standard transformation conditions is given in Materials and Methods.

(i) *Impact of components*

The influence of each factor on plasmid transformation was assessed as follows. DH1 cells were concentrated into transformation buffers at 0°C that were either complete or missing one cation. Cells under each set of conditions were then

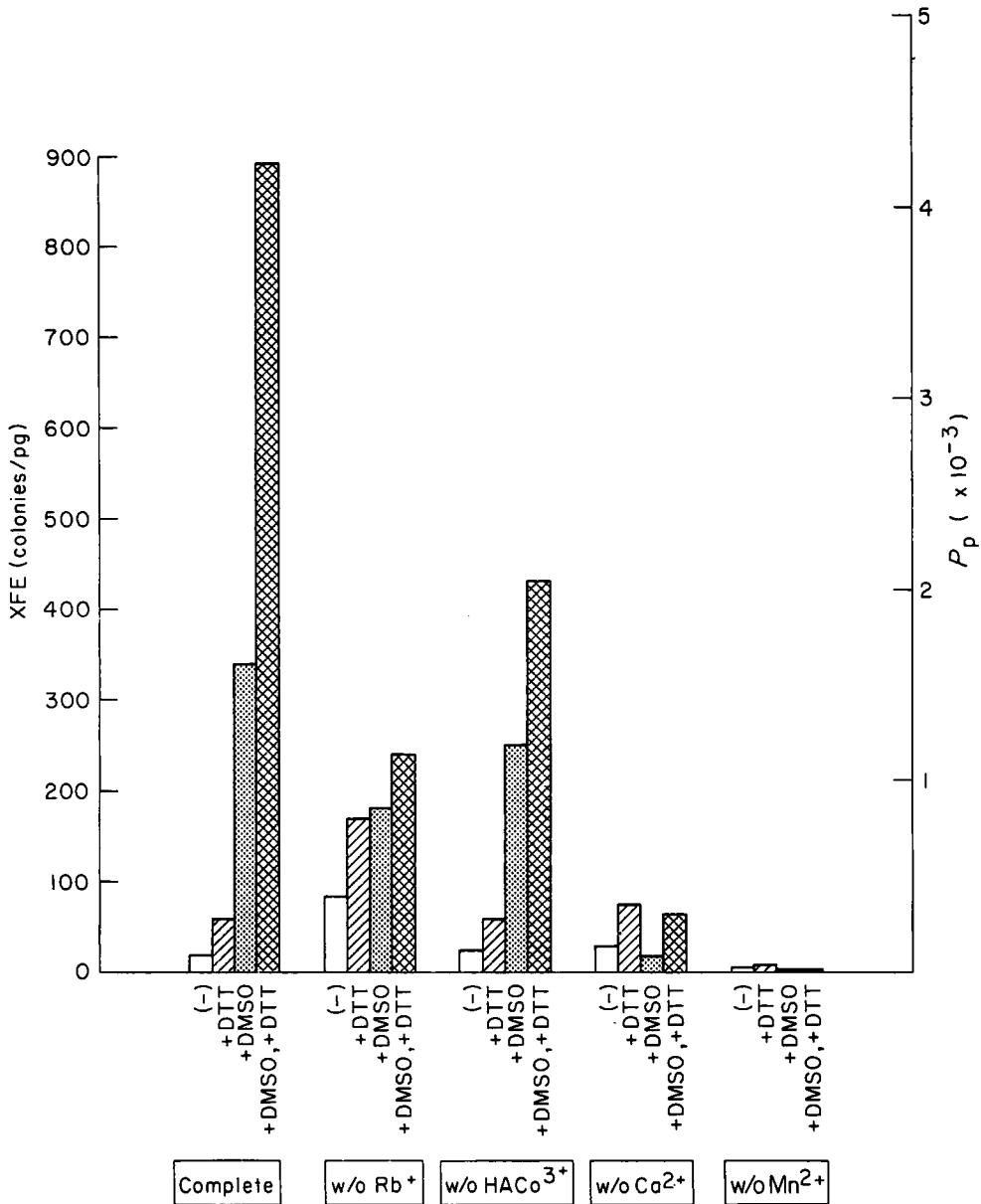


FIG. 1. Impact of components. A series of transformation buffers (TFB) were prepared that were either complete or lacking one cation. Each was employed in a transformation assay in which DMSO, dithiothreitol, both, or neither, were subsequently added.

subsequently treated with dithiothreitol, DMSO, both or neither, and then mixed with 10 pg of pBR322, incubated at 0°C, heat-pulsed for 90 seconds at 42°C, chilled, diluted into growth medium, incubated at 37°C for 60 minutes (to express antibiotic resistance) and spread on selective plates. The results presented in Figure 1 are the consensus of three separate experiments.

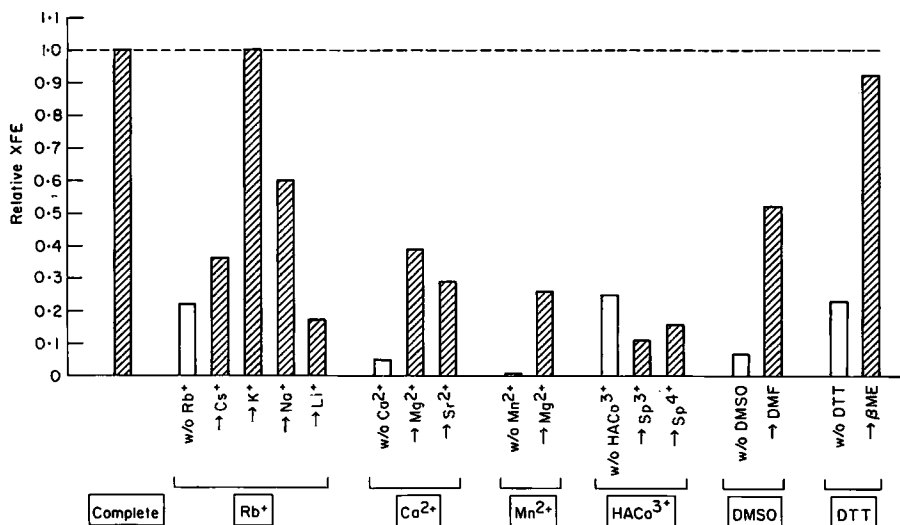


FIG. 2. Specificity of components. Transformations were conducted under conditions including or lacking a particular component. Similar compounds were substituted at the same concentration (except for 2-mercaptoethanol, which was used at 25 mM instead of 75 mM), and the results are given for each substitution (including the null substitution) relative to the complete conditions. DMF, dimethyl formamide.

Divalent cations have a profound effect on the efficiencies of transformation. Absence of Mn^{2+} reduces the transformation probability 500-fold, while lack of Ca^{2+} results in a 15-fold drop. Absence of HAcCo^{3+} , Rb^+ , or DTT, reduces P_p two- to fourfold, while lack of DMSO reduces P_p 15 to 20-fold. Thus, all of these compounds contribute significantly to the conditions under which *E. coli* DH1 is efficiently transformed by pBR322.

Mn^{2+} , Ca^{2+} , Sr^{2+} , and Mg^{2+} were each analyzed at 45 mM levels in 10 mM-K-MES (pH 6.2) in order to evaluate their ability to serve as primary stimulants of transformation (not shown). All four stimulated transformation of DH1 by pBR, and the relative efficiencies were $\text{Mn}^{2+} > \text{Ca}^{2+} \sim \text{Sr}^{2+} \gg \text{Mg}^{2+}$. Thus Mn^{2+} is more effective than Ca^{2+} , both alone and in complex conditions.

(ii) Specificity of components

The specificity of each factor was examined by its substitution with similar compounds and their comparison under identical conditions. Figure 2 shows the consensus of several experiments in which substitutions were compared both to the factor as well as to transformation in its absence. Relative to the removal of a component, many similar compounds substitute reasonably well. K^+ is consistently equivalent to Rb^+ for DH1. Na^+ is also a good substitute, while Li^+ is not. Both Mg^{2+} and Sr^{2+} substitute for Ca^{2+} , and Mg^{2+} substitutes well for Mn^{2+} (again relative to the null substitution). Many divalent cations other than the alkali earth metals were not effective substitutes (e.g. Zn^{2+} , Cd^{2+} , Co^{2+} , Mo^{2+}). The solvent dimethyl formamide is a good substitute for DMSO, and β -mercaptoethanol is a very effective substitute for dithiothreitol. Cysteine will also

substitute for dithiothreitol (not shown). HACo^{3+} is the only factor refractory to substitution with similar compounds. Neither spermine (Sp^{4+}) or spermidine (Sp^{3+}) will substitute; nor will CoCl_2 .

(iii) *Interactions of factors*

Examination of Figure 1 with regard to the stimulation of transformation by the various components suggests some interrelation in their actions. DMSO has no effect in the absence of either Ca^{2+} or Mn^{2+} , and only a minor stimulatory effect without Rb^+ . Conversely, in the absence of DMSO, conditions also lacking Rb^+ are better than those containing it. These observations imply that the actions of Mn^{2+} , Ca^{2+} , Rb^+ and DMSO are, in some sense, interrelated.

Regardless of the basal efficiency, dithiothreitol stimulates transformation in all complex conditions and the comparison holds with and without DMSO (Fig. 1; compare tracks 1 and 2; 3 and 4 of all sets; each pair is — and + dithiothreitol, respectively). The pattern of response to DMSO and dithiothreitol in the absence of HACo^{3+} parallels that in its presence. These comparisons suggest that dithiothreitol and HACo^{3+} each act independently from the other factors, which seem to be acting in a co-operative fashion.

(iv) *Growth conditions*

The effects of different growth media on the development of competent *E. coli* cells were examined. There was no significant difference found upon comparing various rich media (e.g. nutrient broth, tryptone and yeast extract, Casamino acids and yeast extract, etc.). However, concentrations of the components that increased growth rates in a given medium also improved subsequent transformation. This is in accord with the results of Jones *et al.* (1981), using chemostat cultures of *E. coli*.

The presence of 10 to 20 mM- Mg^{2+} in all growth media considerably stimulates transformation efficiency. Cell growth in media with and without 20 mM- Mg^{2+} proceeds at the same rate, but the transformation efficiency is consistently enhanced 15 to 20-fold by the presence of Mg^{2+} (Fig. 3). The levels of Mg^{2+} and Ca^{2+} in 2% Difco Bacto tryptone plus 0.5% Difco yeast extract are <0.5 mM and <0.1 mM, respectively (Difco Labs, unpublished data). Addition of Mg^{2+} 30 minutes before collecting the cells is also stimulatory (~60% of maximal). Furthermore, addition of Mg^{2+} as the cells are collected and placed on ice improves transformation efficiencies to ~40% of that obtained with continual growth in Mg^{2+} . This indicates that Mg^{2+} is not inducing or repressing synthesis of some factor but rather modifies or stabilizes pre-existing components. Addition of Ca^{2+} or Mn^{2+} as the cells are collected also stimulates transformation, so this is a general effect of divalent cations.

(v) *Cell density*

Competence for transformation is characteristically a transient phenomenon in bacteria. In *E. coli*, the period of maximal competence in growth occurs mid to late in the logarithmic phase, at cell densities of 10^7 to 10^8 /ml (Taketo, 1974;

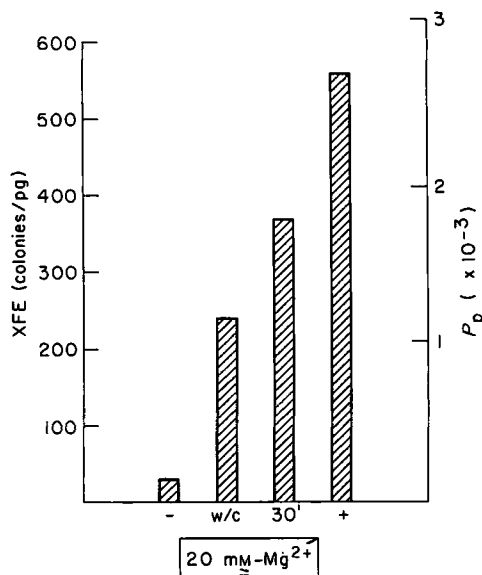


FIG. 3. Effects of Mg^{2+} during growth. Four flasks of medium were inoculated with 1/4 of several colonies of DH1 that were dispersed in SOB medium ($-Mg^{2+}$). Three flasks contained SOB medium ($-Mg^{2+}$), and the 4th SOB medium ($+20\text{ mM-}Mg^{2+}$). The flasks were incubated until the cell density was $\sim 5 \times 10^7/\text{ml}$. The cultures were collected, placed on ice, and subjected to standard transformation conditions. One culture was made $20\text{ mM-}Mg^{2+}$ 30 min before being collected (denoted 30 min), another made 20 mM in Mg^{2+} as the cells were collected and placed on ice (denoted w/c). One culture never saw high Mg^{2+} ($-$), and the 4 grew continuously in Mg^{2+} ($+$).

Norgard *et al.*, 1978). In the course of many experiments on the strains $\chi 1776$ and DH1, using the transformation buffer lacking dithiothreitol and $HACo^{3+}$, a sharp peak in competence was observed at 5×10^7 to 7×10^7 cells/ml. This peak (100 to 400 colonies/pg) appeared in 20 to 40% of the individual preparations of competent cells, and all attempts to produce it routinely failed.

The development of competence during growth of DH1 was examined using transformation conditions with and without dithiothreitol and $HACo^{3+}$. On four separate days, DH1 cells were inoculated into growth medium under identical circumstances and incubated. Cells were collected at several points during mid to late log phase and chilled. The concentration of viable cells was determined by plating serial dilutions. The chilled cells were divided into two equal portions and carried through the standard transformation procedure. One portion was treated under conditions that included dithiothreitol and $HACo^{3+}$, while the other was treated under identical conditions except that dithiothreitol and $HACo^{3+}$ were omitted. The results are shown in Figure 4. It can be seen that the sharp peak in competence described previously is present on one of the the four days (Δ). The effects of dithiothreitol and $HACo^{3+}$ are both to increase overall transformation efficiencies and to widen the range of cell densities that produce those levels. The sharp peak in competence is not observed in the conditions including dithiothreitol and $HACo^{3+}$. It has apparently been expanded to cover most of the mid-log phase of growth, and is now reproducible in virtually every preparation of

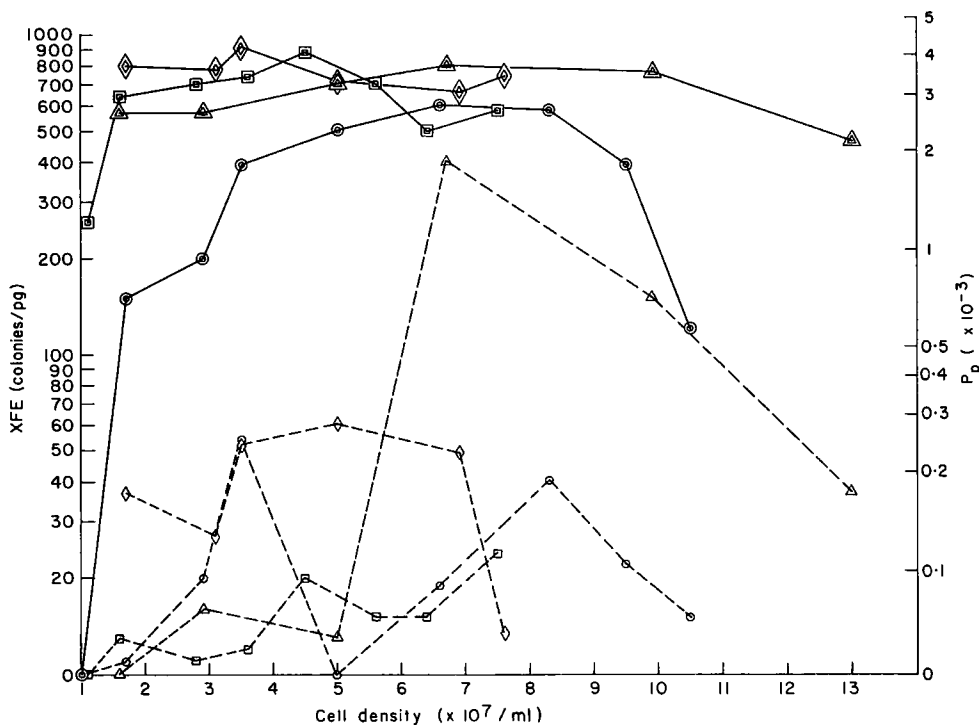


FIG. 4. Transformation efficiency and cell density. On 4 days DH1 cells were incubated in SOB medium and collected at several points during growth. One half of each sample was subjected to transformation conditions including H_4CO_3^+ and dithiothreitol and the other half to conditions lacking both. Samples were adjusted so that the cell concentrations in the transformation conditions were approximately equal ($\sim 6 \times 10^8/\text{ml}$). Double boxes indicate conditions including H_4CO_3^+ + dithiothreitol; single boxes those lacking both. The form of the symbol denotes the day (e.g. \odot are one day, with and without).

cells. Further experiments separated the additions of dithiothreitol and H_4CO_3^+ (not shown), and these indicate that H_4CO_3^+ is primarily responsible for broadening the range of cell densities giving maximal transformation efficiencies, while both increase efficiencies independently.

The combination of all these factors both in growth and uptake conditions significantly improves the efficiency with which plasmids transform *E. coli*. Using pBR322, efficiencies of 5×10^8 transformants per microgram of plasmid can be obtained routinely. This corresponds to one transformed cell per 400 plasmid molecules, and represents a 100 to 1000-fold improvement over levels observed under the standard conditions using Ca^{2+} at 0°C .

(b) Characteristics of plasmid transformation

The improvements in transformation efficiency and the factors that underly it must eventually reflect upon the mechanisms of DNA transfer into the cell. So too must the characteristics of the process, and several of these have been examined and are described below.

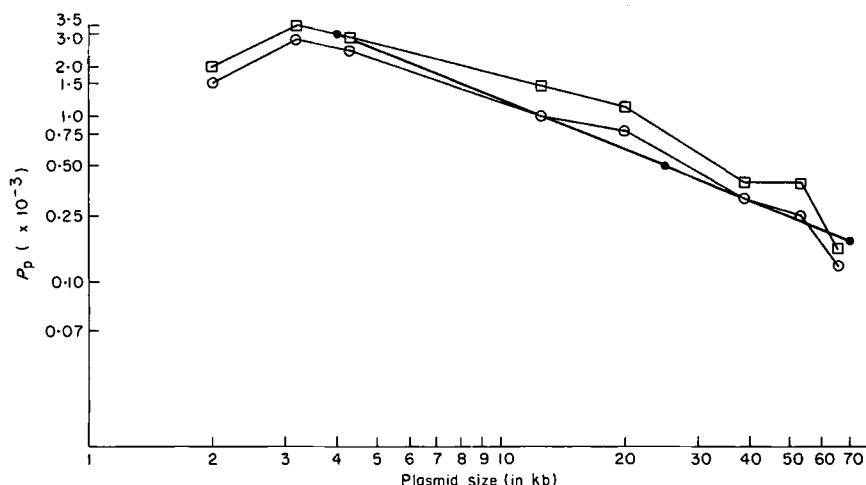


FIG. 5. Transformation probabilities of relaxed and supercoiled plasmids. Plasmids were assayed under the standard transformation conditions to determine the characteristic transformation probability of both their supercoiled (□) and relaxed (○) forms. The plasmids were: pXf1, 2 kb, see Table 1; pXf3, 3.2 kb, see Table 1; pBR, 4.3 kb; pV, 12.5 kb, chicken collagen sequences in pBR; p20, 20 kb, random chicken DNA in pBR; pXAD, 39 kb, a molecular clone of adenovirus 5 in pXF3 (D. Hanahan & Y. Gluzman, unpublished results); RP4, 54 kb, a naturally occurring plasmid; and p66, 66 kb, human globin sequences in pBR (P. Charnay, personal communication).

(i) Effects of size and supercoiling

If plasmids are entering *E. coli* cells by passive diffusion, then one might expect that the compact supercoiled form of a given plasmid would transform much more readily than its extended open circular form. Furthermore, the transformation probability should decline sharply as the dimensions of a plasmid approach those of the pore. A series of plasmids ranging from 2 kb to 66 kb were tested to determine their characteristic transformation probability (P_p). All but one (RP4) are recombinant DNAs derived from pBR322 and carry its origin of replication and at least one of its antibiotic resistance genes. All the plasmid DNAs were purified by banding on cesium chloride density gradients and chromatography through Biogel A50f columns. Relaxed plasmids were prepared by treatment with calf thymus topoisomerase I. Both supercoiled and relaxed versions of each plasmid were used in transformation experiments; the results are shown in Figure 5.

The relaxed form of each plasmid transforms about 75% as well as its supercoiled form, and this correlation holds across the size range of 2 kb to 66 kb. The transformation probability P_p decreases linearly with increasing plasmid size. The straight line in Figure 5 describes a linear decay in P_p , given by the relation $P_p[n \text{ kb}] = P_p[4 \text{ kb}] \times (4 \text{ kb})/(n \text{ kb})$, shown on a log scale. No sharp cutoff in transformation probability is observed, even with a 66 kb plasmid, whose contour length is $\sim 20 \mu\text{m}$. The dimensions of an *E. coli* cell are roughly $1 \mu\text{m}$ in diameter $\times 2 \mu\text{m}$ long. These results are not consistent with the predictions of a simple model involving passive diffusion of plasmids through large pores in the cell.

TABLE 1
Transformation probabilities: pBR derivatives and compatible plasmids

Plasmid	Size (kb)	$P_p (\times 10^{-3})$	Description	Reference
pBR322	4.3	2.5	<i>amp^r tet^r</i> ColE1 origin	Bolivar <i>et al.</i> (1977)
pXf1	2.0	1.6	<i>tet^r</i> pBR origin (ThaA) [(1-1425) + (2521-3102)]	This paper
pXf6	2.0	2.8	<i>tet^r</i> , high copy number [(1-1425) + (2449-2845) + (2884-3090)]	This paper
pXf8	1.8	1.9	<i>amp^r</i> , high copy number [(3044-4300) + (2521-2845) + (250 bp undefined)]	This paper
pXf3	3.2	2.6	<i>amp^r tet^r</i> , pBR origin (ThaA) [(1-1425) + (2521-3102) + (3223-4361)]	This paper
pXf7	3.2	2.6	<i>amp^r tet^r</i> , high copy number [(1-1425) + (2449-2845) + (2884-3090) + (3223-4361)]	This paper
RSF1030	7.0	1.0	<i>amp^r</i> , compatible <i>ori</i> (RSF)	Crosa <i>et al.</i> (1975)
pAC184	4.0	1.6	<i>cam^r tet^r</i> , compatible <i>ori</i> (pAC)	Chang & Cohen (1978)

The pXf series of plasmids were constructed using standard techniques for recombinant DNA. The parentheses refer to the sequence co-ordinates of the pBR322 fragments used in each construction (Sutcliffe, 1979).

(ii) Assessments of DNA sequence specificity in uptake

The demonstration of sequence-specific uptake of DNA in the gram-negative bacteria *Haemophilus influenzae* (Scocca *et al.*, 1974; Sisco & Smith, 1979; Chung & Goodgal, 1979) provokes questions of similar effects in *E. coli*. This has been addressed in two ways: by comparing deletion derivatives of pBR322 and by competing for pBR uptake with excesses of non-transforming DNAs.

Derivatives of pBR322 were constructed that remove all excess DNA from the plasmid. pXf1 consists of the origin of replication (the *ThaA* fragment) plus the *tet^r* gene. pXf3 includes the *amp* gene. pXf6 and pXf8 carry *tet* and *amp*, respectively, and share a mutant origin of replication. This origin carries a deletion that removes a putative DNA binding site (Backman *et al.*, 1979) as well as half of the transcription unit for the small RNA that regulates plasmid copy number (Morita & Oka, 1979; Itoh & Tomizawa, 1980). pXf7 includes both *amp* and *tet* with this mutant origin. All three have very high copy numbers.

Transformation probabilities of these plasmids are given in Table 1. No significant difference is observed among the various plasmids. The only sequences in common to all are the two small fragments comprising the origin of replication. Thus, it can be concluded that there is no single copy sequence distinct from the origin that affects transformation by pBR. Two other plasmids (pAC184 and RSF1030), which carry different origins of replication (defined both by heteroduplex analysis and plasmid compatibility), show similar transformation probabilities (Table 1).

In order to examine the effects of added non-transforming DNA, both linear and supercoiled DNAs from various sources were used in mass ratios of 500 × to

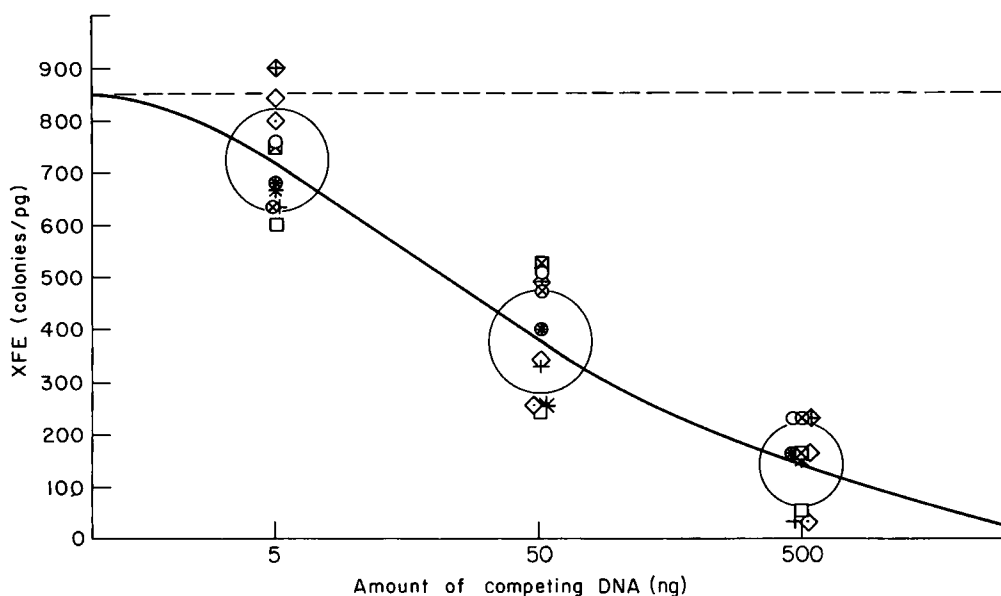


FIG. 6. Competition with non-transforming DNAs. Various DNAs were used at 5 ng, 50 ng and 500 ng amounts to compete with 10 pg of pBR322 in a saturated transformation assay. The curve is drawn through the average values at each mass ratio, the circles indicate the standard deviation from the average, and the broken line marks the transformation efficiency without competing DNA. The DNAs were: pXfl (supercoiled) \diamond ; π VX (supercoiled) \oplus ; SV40 (supercoiled) \diamond ; human placenta (HMW) \square ; *E. coli* DH1 \otimes ; pXfl (*Hinf*) \oplus ; π VX (*Hinf*) \circ ; SV40 (*Hinf*) $*$; human placenta (*Hinf*) \boxtimes ; and *E. coli* DH1 (*Hinf*) \otimes .

50,000 \times to compete with ten picograms of pBR322 in transformation. The complexity ranged from a 900 bp miniplasmid (π VX) to human DNA. The sources of *Hinf*-cleaved low molecular weight DNAs were DH1 *E. coli* DNA, π VX, pXfl, simian virus 40 (SV40), and human placental DNA. High molecular weight linear DNAs were DH1 *E. coli* DNA and human placental DNA. Supercoiled DNAs were π VX, pXfl, and SV40. Figure 6 shows the results of competition experiments performed with each of these DNAs. It is clear that non-transforming DNA competes according to mass only, and no significant effect of complexity, source, or form (linear *versus* supercoiled; low molecular weight *versus* high molecular weight) is observed. The results with π VX are of special interest, for it is essentially a clone of the pBR origin of replication (B. Seed, personal communication). Yet the results obtained with it and with pXfl indicate that neither compete differently from the other DNAs. The plasmid pMB9, a larger progenitor of pBR322, also competes for pBR transformation in an analogous fashion to π VX and pXfl (not shown). π VX at 500 ng represents a 2.5×10^5 molar excess of origins of replication competing with each pBR origin; pXfl represents a 10^5 molar excess (in this experiment, pBR was selected for ampicillin resistance, so pXfl is non-transforming). DH1 DNA competes about as well as human DNA. These comparisons, along with those of the deletions of pBR described above, indicate that there is no sequence specificity in DNA uptake by *E. coli*, carried either in *E. coli* DNA or on the plasmids.

TABLE 2
Linearity of response

Amount of pBR	Number of plasmid molecules N_p	Number of trans- formed cells (colonies) N_{XF} ($\sigma < 0.2X$)	Plasmid-to-cell ratio N_p/N_c	Probability of a plasmid transforming a cell $P_p = N_{XF}/N_p$ ($\sigma < 0.2X$)
A. <i>DH1</i> rec ⁻ , 1.2×10^8 viable cells/xf				
500 ng	1×10^{11}	3.0×10^6	830/1	3.0×10^{-5}
300 ng	6×10^{10}	3.3×10^6	500/1	5.0×10^{-5}
200 ng	4×10^{10}	4.4×10^6	330/1	1.0×10^{-4}
100 ng	2×10^{10}	2.7×10^6	165/1	1.5×10^{-4}
35 ng	7×10^9	2.1×10^6	58/1	3.0×10^{-4}
10 ng	2×10^9	1.7×10^6	17/1	8.5×10^{-4}
3.5 ng	7×10^8	1.3×10^6	6/1	2.0×10^{-3}
1 ng	2×10^8	5.0×10^5	2/1	2.5×10^{-3}
100 pg	2×10^7	4.6×10^4	1/6	2.3×10^{-3}
10 pg	2×10^6	4500	1/60	2.3×10^{-3}
1 pg	2×10^5	440	1/600	2.2×10^{-3}
100 fg	2×10^4	32	1/6000	1.6×10^{-3}
10 fg	2×10^3	3	$1/6 \times 10^4$	1.5×10^{-3}
B. <i>M294</i> rec ⁺ , 2×10^8 cells/xf				
300 ng	6×10^{10}	2.3×10^6	300/1	3.8×10^{-5}
200 ng	4×10^{10}	2.2×10^6	200/1	5.5×10^{-5}
100 ng	2×10^{10}	1.9×10^6	100/1	9.5×10^{-5}
10 ng	2×10^9	1.2×10^6	10/1	6.0×10^{-4}
5 ng	1×10^9	1.1×10^6	5/1	1.1×10^{-3}
4 ng	8×10^8	1.0×10^6	4/1	1.3×10^{-3}
3 ng	6×10^8	8.3×10^5	3/1	1.4×10^{-3}
2 ng	4×10^8	5.1×10^5	2/1	1.3×10^{-3}
1 ng	2×10^8	4.2×10^5	1/1	2.1×10^{-3}
500 pg	1×10^8	2.1×10^5	1/2	2.1×10^{-3}
250 pg	5×10^7	9.6×10^4	1/4	1.9×10^{-3}
100 pg	2×10^7	3.9×10^4	1/10	2.0×10^{-3}

(iii) *Linearity of response*

The response of the transformation process to different numbers of plasmid molecules was examined by adding variable amounts of DNA to identical quantities of cells in transformation conditions. Each separate transformation contained $\sim 1.2 \times 10^8$ viable cells in a volume of $220 \mu\text{l}$ ($\sim 5.5 \times 10^8$ cells/ml). Table 2A gives the response to plasmid DNA ranging from 10 fg to 500 ng (2×10^3 to 1×10^{11} molecules). The transformation probability is essentially linear between 1 pg and 1 ng, and tails off only slightly in the femtogram range. Above plasmid to viable cell ratios of 2 : 1, P_p begins to fall off, and the system becomes saturated at ~ 200 ng pBR (plasmid to cell ratio = 330 : 1). Under conditions of plasmid excess, $\sim 3.5\%$ of the viable cells became transformed ($F_c = 0.035$). A linear response has been observed using the conditions of $\text{Ca}^{2+}/0^\circ\text{C}$, although P_p , F_c and the saturation levels were all different (Taketo, 1972; Cohen *et al.*, 1972; Weston *et al.*, 1981).

A characteristic of recombination deficient strains is the presence of non-viable cells in the population, which are presumed to arise from aberrant repairs of DNA

TABLE 3
Competition between compatible plasmids

Number ratio pBR/pAC'	Number of double transformants (<i>amp</i> + <i>cam</i>) $N_d (\times 10^5)$	Number of single transformants	
		$N_{amp} (\times 10^5)$	$N_{cam} (\times 10^5)$
1/5	5 ± 2	3 ± 2	8 ± 2
1/3	5 ± 2	—	—
1/2	4 ± 2	—	—
1/1	5 ± 2	6 ± 2	6 ± 2
2/1	4 ± 2	—	—
3/1	4 ± 2	—	—
5/1	3 ± 2	9 ± 3	3 ± 2
11/1	2.5 ± 1.5	—	—
30/1	1.5 ± 0.5	—	—
85/1	0.7 ± 0.2	—	—
300/1	0.22 ± 0.04	10 ± 3	—

Number of viable cells $N_c = 1.0 \times 10^8/\text{xf}$.

Amount of DNA = 300 ng/xf.

XFE (10 pg) = 2×10^8 colonies/ μg .

chain breaks that occur during growth. A population of cells carrying the *recA* allele generally contains 50% non-viable cells (Capaldo-Kimball & Barbour, 1971; Capaldo *et al.*, 1974). In order to compare the effects of *recA*⁺ and *recA*⁻ populations, the linearity experiments were repeated with MM294, the *rec*⁺ progenitor of DH1 (Table 2B). The transformation probability becomes non-linear above plasmid-to-cell ratios of 1 : 1, and saturation occurs at 100 to 200 plasmids per cell. Thus in both cases (*rec*⁺ and *recA*), transformation is linear over a wide range, and becomes non-linear as the number of plasmid molecules exceeds the number of cells.

(iv) *Transformation with two compatible plasmids*

In order to assess the frequency of double transformation or cotransformation, experiments were conducted using pBR and pAC184, two distinguishable plasmids that can co-exist stably in an *E. coli* cell. These two plasmids were mixed at differing ratios and transformed under conditions of DNA saturation in order to measure the frequency of cotransformation. The results of one such experiment are shown in Table 3. The cotransformation frequency is seen to be very high. About 1% of the cells became transformed, and 70 to 90% of these were doubly transformed at equimolar ratios. Thus these cells must represent a special class of the total cell population that is competent for plasmid transformation. Otherwise, the double transformation frequency should be the square of the single transformation frequency (or 0.01%). Furthermore, each competent cell must be taking up and establishing more than one plasmid molecule. At ratios of pBR to pAC184 ranging from 1 : 5 to 5 : 1, at least 30% of the transformed cells are doubly transformed. At ratios of 300 : 1, 2% of the cells are doubly transformed. The results indicate that multiple plasmids are taken up under saturating conditions.

TABLE 4
Transformation efficiencies of *Escherichia coli* strains

Strain	Average XFE (colonies/g)	Genotype	Reference
MM294	5×10^8	F^- , <i>endA1</i> , <i>hsdR17</i> (r_k^- , m_k^+), <i>supE44</i> , <i>thi-1</i> , λ^-	Meselson & Yuan (1968)
DH1	5×10^8	F^- , <i>endA1</i> , <i>hsdR17</i> (r_k^- , m_k^+), <i>supE44</i> , <i>thi-1</i> , λ^- , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> ?	This paper
DH20	4×10^8	DH1/ F' <i>lacI</i> ^{sa} , <i>lacZ</i> ⁺ , <i>proA</i> ⁺ , <i>proB</i> ⁺	This paper
DH21	4×10^8	DH1/ F' <i>lacI</i> ^{sa} , <i>lacZ</i> ⁺ , <i>proA</i> ⁺ , <i>proB</i> ⁺	This paper
RR1	1×10^8	F^- , <i>hsdS20</i> (r_B^- , m_B^-), <i>supE44</i> , <i>ara-14</i> , <i>galK-2</i> , <i>lacY1</i> , <i>proA2</i> , <i>rpsL20</i> (<i>str</i> ^R), <i>xyl-5</i> , <i>mtl-1</i> , λ^-	Bolivar <i>et al.</i> (1977)
HB101	1×10^8	F^- , <i>hsdS20</i> (r_B^- , m_B^-), <i>supE44</i> , <i>ara-14</i> , <i>galK-2</i> , <i>lacY1</i> , <i>proA2</i> , <i>rpsL20</i> (<i>str</i> ^R), <i>xyl-5</i> , <i>mtl-1</i> , λ^- , <i>recA13</i>	Boyer & Rouland-Dussoix (1969)
C600	5×10^8	F^- , <i>supE44</i> , <i>thi-1</i> , <i>thr-1</i> , <i>leuB6</i> , <i>lacY1</i> , <i>tonA21</i> , λ^-	Appleyard (1954)
BJ5183	5×10^8	F^- , <i>endA</i> , <i>sbcB</i> ⁻ , <i>recBC</i> ⁻ , <i>galK</i> , <i>met</i> ⁻ , <i>str</i> ^R , <i>thi-1</i> , <i>bioT</i> , <i>hsdR</i> (r_k^- , m_k^+), λ^-	D. Beech
LE392	3×10^8	F^- , <i>hsdR514</i> (r_k^- , m_k^-), <i>supE44</i> , <i>supF58</i> , <i>lacY1</i> or ∇ (<i>lacIZY</i>)6, <i>galK2</i> , <i>galT22</i> , <i>metB1</i> , <i>trpR55</i> , λ^-	L. Enquist
W5449	2×10^8	F^- , <i>hsdR18</i> , <i>supE44</i> , <i>recB21</i> , <i>recC22</i> , <i>sbcB15</i> , <i>tonB56</i> , <i>tsx-33</i> , <i>ara-14</i> , <i>argE3</i> , <i>galK2</i> , <i>his4</i> , <i>lacY1</i> , <i>leuB6</i> , <i>mtl-1</i> , <i>proA2</i> , <i>rpsL31</i> , <i>xyl-5</i> , <i>trpB9579</i> , <i>thi-1</i> , λ^-	Sgaramella <i>et al.</i> (1976)
SK2267	2×10^7	F^- , <i>endA1</i> , <i>hsdR4</i> , <i>supE44</i> , <i>thi-1</i> , λ^- , <i>lacZ4</i> or <i>lac-61</i> , <i>gal-44</i> , <i>ton-58</i> , [<i>rfa</i>], <i>recA1</i> , <i>sbcB15</i>	Kushner (1978)
JM103	1×10^8	<i>endA</i> , <i>hsdR</i> , <i>supE</i> , <i>sbcB</i> , <i>thi-1</i> , <i>strA</i> , ∇ (<i>lac-pro</i>), λ^- / F' <i>traD36</i> , <i>proAB</i> ⁺ , <i>lacI</i> ^{sa} , ZM15	Messing <i>et al.</i> (1981)

Frozen stocks of each strain were prepared. Fresh colonies derived from each were used to initiate a standard transformation assay, in which the cells were collected at about 5×10^7 cells/ml and transformed by 10 pg of pBR322. The averages are derived from transformation on at least 2 separate occasions.

(v) Comparison of *Escherichia coli* strains

A considerable number of different strains of *E. coli* have been examined for their susceptibility to transformation by pBR in the standard transformation assay. Every strain tested has been observed to yield higher transformation probabilities under these conditions than with 50 mM-Ca²⁺. However, there are consistent differences between strains. Twelve representative strains are described in Table 4. Several transform as readily as DH1. There is no obvious genetic distinction among the strains that correlates with differences in their characteristic transformation efficiencies. In particular, in the two cases of isogenic *rec*⁺/*recA*⁻ strains (MM294/DH1; RR1/HB101), no difference is observed in transformation efficiency. In addition, the presence of F-factors and their associated conjugation apparatus (F pili, etc.) does not significantly affect transformation. The comparison of defined markers other than *F'* and *recA* in these strains gives no consistent relation (e.g. *endA1* or *galK*), although definitive

proof awaits comparison between otherwise isogenic strains. In summary, the conditions described in this paper are applicable to a variety of *E. coli* strains.

4. Discussion

An evaluation of factors previously reported to affect DNA transfer into *E. coli* and a search for other stimulants has resulted in the development of conditions in which plasmid DNA transforms *E. coli* at much higher frequencies than heretofore observed. These conditions have proved to be applicable to most *E. coli* K12 strains. Cells are collected in the mid-logarithmic phase of growth in rich medium containing elevated levels of Mg^{2+} , and combined with DNA at 0°C in an environment comprised of Mn^{2+} , Ca^{2+} , Rb^+ or K^+ , HACo^{3+} , DMSO, and dithiothreitol. The influence of these factors has been examined by removing each from the protocol and by substituting each with similar compounds. Divalent cations are readily substituted among the alkali earth metals and Mn^{2+} , and there is no absolute requirement for Ca^{2+} . The monovalent cation is necessary but non-specific. Both the solvent and the sulfhydryl reagent can be interchanged with compounds possessing similar activities. Only HACo^{3+} is refractory to substitution, and hence apparently quite specific. Mn^{2+} , Ca^{2+} , Rb^+ and DMSO appear to act in a co-operative manner, as the removal of any one considerably reduces the efficacy of those remaining.

(a) *Size and superhelicity*

The probability P_p that a plasmid will transform a cell declines linearly with increasing size. Relaxed plasmids transform at 75% of the efficiency of their supercoiled forms, over a size range of 2 kb to 66 kb. The linear decay in P_p and the lack of a cutoff in transformation associated with increasing plasmid size, as well as the absence of a significant distinction in transformation between relaxed and supercoiled forms of a plasmid, all argue against a simple view of uptake in which plasmids diffuse through holes in the cell.

(b) *Specificity and channels in transformation*

Competition experiments with non-transforming DNAs indicate that there is no sequence specificity in plasmid transformation of *E. coli*. DNAs varying in source, complexity, length and form (linear or circular) all compete consistent with mass. Deletion derivatives of pBR that combinatorially remove everything but the origin of replication and have both normal and very high copy numbers were all found to transform with probabilities similar to pBR, as do plasmids with different origins of replication.

The dose response is nearly linear over a range of 10^4 , from plasmid-to-cell ratios of 1 : 2000 to 1 : 1. Thus a single plasmid is sufficient to transform a cell. In the *rec*⁺ strain MM294, the transformation probability becomes non-linear above one plasmid per cell, and saturation occurs at ~150 plasmids per cell. Transformation of the *rec*⁻ strain DH1 becomes non-linear above two plasmids per viable cell, and saturation occurs above ~300 plasmids per viable cell. Since

only 50% of the cells in a *recA* population are viable, comparison of these results suggests that all cells compete for plasmids. Increasing the plasmid-to-cell ratios 200-fold, from 1 : 1 to 200 : 1, only increases the number of transformed cells by six to eightfold. Thus, providing the cells with multiple plasmids does not significantly improve their transformation.

Competition for pBR transformation (to *amp^r*) with the pBR derivative pXfl (*amp^s*) at 1000 : 1 ratios of pXfl to pBR and plasmid-to-cell ratios of 10 : 1 results in no inhibition of P_{pBR} . When the plasmid-to-cell ratio is increased to 100 : 1 (and pXfl : pBR is 10,000 : 1), P_{pBR} only drops to 40% of its maximal value, and when the competition is increased still further, to 1000 plasmids per cell (pXfl : pBR = 100,000 : 1), P_{pBR} is 20% of that observed without competing DNA. Similarly, when competing with pBR for AC184 transformation (to *cam^r*) at levels of 250 plasmids per cell (pBR : pAC = 300 : 1), P_{pAC} is 10% of its maximal value.

An interpretation consistent with these results is that there are many sites or channels on the cell that the plasmids initially associate with, and each of these sites must have an independent probability of participating in uptake and subsequent transformation. There must be more than ten channels, since plasmid-to-cell ratios of 10 : 1 do not result in pXfl competition for pBR transformation. And there are probably less than 200 channels, as a plasmid-to-cell ratio of 200 : 1 will saturate all the transformable cells. Furthermore, the pXfl competition results, in which 1000 plasmids per cell dropped P_{pBR} to 20% of its unchallenged value, is consistent with 1000 plasmids competing for several hundred sites on a cell. An additional implication of these results is that all cells are competing for plasmids. If only competent cells competed, the effective plasmid-to-cell ratios would be some 50-fold higher, which is incongruous with the characteristics of the competition.

If one considers the transformation process to be divisible into phases of uptake and establishment, then both transformation probabilities (P_p and F_c) will include factors describing each phase. The competition and cotransformation experiments indicate that there are a number of independent channels that can each lead to transformation with similar probabilities. If several independent channels are occupied with identical plasmid molecules, the uptake factor in the probability (F_c) that a cell will become transformed should be the sum of the probabilities of uptake through each channel. However, the linearity experiments demonstrate that providing a cell with increasing numbers of plasmids does not improve its prospects for transformation in an additive manner. Therefore, uptake must be very efficient, with establishment then being the limiting process, one that is not strongly affected by the number of plasmid molecules present.

(c) *Modes of action of divalent cations and DMSO*

There are several possible roles for divalent cations and DMSO; all involve ionic interactions with phosphate moieties. The interaction of the cell with exogenous DNA is similar to that of two complex polyanions: the DNA with its phosphate backbone, the cell with a surface of phospholipid and lipopolysaccharide (itself a

phospholipid). Divalent cations are adept at shielding phosphates, and form much more stable co-ordination complexes with phosphate than do monovalent cations (Sillen, 1964). DMSO is very effective at solvating metallic cations (Meek *et al.*, 1960; David, 1972), and should be particularly effective at organizing and stabilizing ionic interactions along boundaries between hydrophobic lipid bilayers and the aqueous environment. Thus these compounds may be facilitating the otherwise unlikely association of two phosphate-rich structures.

The *E. coli* cell envelope is comprised of a cell wall (the peptidoglycan) separating two phospholipid membranes (Costerton *et al.*, 1974; DiRienzo *et al.*, 1978; Osborn & Wu, 1980). There are approximately 400 zones of adhesion, where the cytoplasmic and outer lipid bilayers are fused through holes in the peptidoglycan (Bayer, 1968; Muhlradt *et al.*, 1973). At low temperatures, both membranes undergo transitions, in which the phospholipids cease to rotate, twist and diffuse, and instead become closely packed and behave as a solid (Overath *et al.*, 1975). Millimolar concentrations of divalent cations and temperatures near 0°C are essential for producing similar phase transitions in synthetic membrane vesicles (Verkleij *et al.*, 1974; Papahadjopoulos *et al.*, 1977). Thus low temperatures, divalent cations and DMSO may together induce phase transitions that crystallize regions of the membranes, thereby affecting the conformation and accessibility of channels through the envelope.

The lipopolysaccharide is a long chain of saccharide molecules linked to a phosphorylated disaccharide core, off which phospholipids extend, anchoring the molecule into the outer face of the the outer membrane. Complexes of lipopolysaccharides and outer membrane proteins are organized around (and probably within) the zones of adhesion, and seem to serve as gate-keeping functions by restricting both the uptake of macromolecules and the efflux of the contents of the cell (Leive, 1974; Osborn & Wu, 1980). About 40% of the lipopolysaccharides covering the cell can be stripped off with 1 mM-EDTA (Leive, 1974). When *E. coli* cells are grown or incubated in 10 to 20 mM-Mg²⁺ or Ca²⁺, about 70% of the lipopolysaccharides become subject to removal by treatment with EDTA (Leive, 1974). Thus high levels of divalent cations appear to reorganize the lipopolysaccharide. The observation that the presence of 20 mM-Mg²⁺ or Ca²⁺ during growth also improves transformation efficiency suggests that a third role for divalent cations is to reorient the lipopolysaccharides, perhaps away from the channels they are protecting, or into a different conformation.

(d) *Hexamine cobalt (III) chloride—an analog of cobalamin?*

There are two attractive roles for HAcCo³⁺ in transformation. It could either be affecting the conformation of DNA, or acting as an analog of cobalamin (vitamin B₁₂), an organo-cobalt (III) compound. HAcCo³⁺ is capable of producing conformational changes in DNA (Widom & Baldwin, 1980; Behe & Felsenfeld, 1981). However, the high concentrations of monovalent salt used here should preclude such conformational transitions (Widom & Baldwin, 1980). Preincubation of DNA in complete transformation buffer or in any subset of it either has no impact or reduces transformation efficiencies (not shown), while

preincubation of the cells in transformation buffer is essential to its action. In addition, both spermine and spermidine can induce similar conformational changes in DNA but neither will substitute for hexamine cobalt in plasmid transformation (cell viability was not affected by these substitutions). Thus it appears that the primary role of hexamine cobalt (III) involves an interaction with the cell.

Cobalamin is not synthesized by *E. coli* or required for growth of wild-type strains, yet a specific active transport system exists for cobalamin uptake (Bassford & Kadner, 1977; Sennet *et al.*, 1981). Cobalamin is of the same order of size as the cross-section of a DNA molecule. Experiments in progress indicate that both vitamin B₁₂ and coenzyme B₁₂ (a derivative of cobalamin carrying an additional adenosine moiety) are potent competitive inhibitors of plasmid transformation. There are about 200 cobalamin receptors per cell, and each receptor appears (by bacteriophage binding) to be associated with a distinct zone of adhesion or uptake channel (Bradbeer *et al.*, 1976). This corresponds roughly to the number of DNA uptake channels defined here by competition experiments. Therefore, it is attractive to suggest that HAcCo³⁺ serves as an analog of cobalamin, either by activating or interfering with components of the cobalamin transport system.

(e) Conclusion

The conditions described here appear to render DNA uptake very efficient, such that virtually every plasmid molecule that interacts with a channel becomes transported across the cell envelope. Events occurring after uptake (establishment) become the limiting step and probably determine the competence for transformation that is observed in a fraction of the cells. Thus, competence in *E. coli* appears to be manifested in a much different manner than in the other bacteria that have been examined, where competence is defined as the ability to recognize and transport DNA into the cell, using a transiently expressed DNA transport apparatus (Smith *et al.*, 1981). DNA transfer across the *E. coli* cell envelope may well involve a specific transport system, either for DNA itself, or for another substrate, with the transport apparatus distorted by the conditions to accept DNA. The characteristics of transformation described in this paper argue against passive diffusion, yet the nature of the components participating in uptake remain undefined.

This discussion has not addressed the mechanisms by which transferred DNA is established in the cell. There are no indications from the observations reported here as to what the mechanism of establishment might be, or how features of it determine competence and affect the low probability of establishing an efficiently transferred plasmid. Possibilities include the involvement of cell cycle, initiation of replication on naked DNA, the organization of the DNA into a "chromatin-like" configuration, or sequestering of transferred DNA.

The improvements in transformation described here have been applied to the rescue of plasmids from mammalian cells (Hanahan *et al.*, 1980; Perucho, *et al.*, 1980a,b; DiMaio *et al.*, 1982), to complementary DNA cloning (Helfman *et al.*, 1983; Tate *et al.*, 1983; Fiddes & Hanahan, unpublished results), to the isolation

of an infectious 39 kb plasmid clone of human adenovirus serotype 5 (Hanahan & Gluzman, unpublished results), and are likely to be applicable to the cloning of large fragments of cellular DNAs.

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